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(54) Title: REGULATION OF CARDIAC CONTRACTILITY AND HEART FAILURE PROPENSITY

(57) Abstract
The methods and compositions of the present invention find use in altering expression of PKCα in transgenic animals. The compositions of the invention include isolated transgenic animal cells, transgenic tissue, transgenic animals, and transgenic mice. The transgenic animals of the invention exhibit altered PKCα activity. The methods allow generation of transgenic animals with altered expression of PKCα. The invention allows modulation of cardiac contractility. In particular, the invention provides a method for altering the susceptibility of a transgenic animal to cardiomyopathy. A

transgenic animal of the invention finds use in identifying anti-cardiomyopathic compounds.

REGULATION OF CARDIAC AND HEART FAILURE PROPENSITY GOVERNMENT GRANT INFORMATION This invention was made with Government support under NIH Grant Nos. HL62927, HL26057, and The United States Government has certain rights in this invention.

FIELD OF THE INVENTION This invention relates to modulation of cardiac contractility and cardiomyopathic phenotypes, prevention and treatment of the same, and transgenic mice related to the same.

BACKGROUND OF THE INVENTION Heart failure afflicts an estimated 5 million Americans, with approximately 400,000 new individuals diagnosed each year at an annual cost of over $20 billion (Lloyd-Jones et al. (2002) Circulation 106: 3068-3072). The predominant therapeutic strategy employed over the past two decades has been based on pharmacological manipulation of cardiac contractility (Remme, W. J.

15: 375-377; Felker et al (2001) Anz. J. Heart 142: 393-401; Packer, M. (2001) Am. J. Med. 110 Suppl 7A: Heart failure may be characterized by a progressive loss in contractility, ventricular chamber dilation, increased peripheral vascular resistance, and/or dysregulated fluid homeostasis. Positive inotropic agents were initially employed as a means of enhancing cardiac pump function, yet are now only utilized to acutely bridge patients in severe heart failure since they worsen long-term survival (Felker et al (2001) Am. J. Heart 142: 393-401). More recently, pharmacological blockade receptors has emerged as the favored treatment for heart failure, although it remains uncertain whether or not benefit the myocardium by diminishing cardiac contractility (short-term) or augmenting it (long-term) (Packer, M. (2001) J. Med. 110 Suppl 7A: ; Bristow M. W.

(2000) Circulation 101: 558-569; et al. (2001) Clin. 15: 95- 109). Two other features associated with the failing human heart are a dysregulation in calcium homeostasis and an increase in neuroendocrine agents that signal through both Gaq- and Gas-coupled receptors.

A variety of human diseases and conditions manifested by cardiac abnormalities or cardiac dysfunction may lead to heart failure. Heart failure is a physiological condition in which the heart fails to pump enough blood to meet the circulatory requirements of the body. The study of such diseases and conditions in genetically diverse humans is difficult and unpredictable.

Therefore, there is a need for a model system that facilitates the identification of potential therapeutic agents of cardiomyopathy.

When stimulated by an array of neuro-humoral factors or when faced with an increase in ventricular wall tension, the myocardium undergoes an adaptive hypertrophic response. Cardiac hypertrophy is an adaptive response of the heart to many forms of cardiac disease, including those arising from hypertension, mechanical load, myocardial infarction, cardiac endocrine disorders, and/or genetic mutations in cardiac contractile protein genes. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to heart failure, and sudden death.

The causes and effects of cardiac hypertrophy have been documented, but the underlying mechanisms that couple hypertrophic signals initiated at the cell membrane to the of cardiomyocyte gene expression remain poorly understood. Elucidation of these mechanisms is a central issue in cardiovascular biology and is important for designing new strategies for prevention or treatment of cardiac hypertrophy and

heart failure.

Studies have implicated intracellular as a signal for cardiac hypertrophy. In response to myocyte stretch or increased loads on working heart preparations, intracellular Ca2+ concentrations increase (Marban et al. (1987) Proc. Natl. Acad. Sci. USA 84 : 6005-6009; Bustamante et 17: ; and Hongo et (1995)

J. 269: C690-C697), consistent with a role of Ca2+ in coordinating physiological responses with enhanced cardiac output.

Hypertrophic stimuli result in reprogramming of gene expression in the adult myocardium, such that genes encoding fetal protein isoforms like p-myosin heavy chain (MHC) and a-skeletal actin are up-regulated, whereas the corresponding adult isoforms a-MHC and a- cardiac are down-regulated. The natriuretic peptides, atrial natriuretic factor and b-type natriuretic peptide, which decrease blood pressure by vasodilation and natriuresis, are also rapidly up-regulated in the heart in response to hypertrophic signals. (Komuro and Yazaki (1993)

Rev. Physio. 55: 55-75). The mechanisms involved in coordinately regulating these cardiac genes during hypertrophy are unknown.

A number of signaling molecules have been characterized as important transducers of this disease response sequelae, including, but not limited to, specific G-protein isoforms, low molecular weight GTPases (Ras, RhoA, Rac), mitogen-activated protein kinases (MAPK), protein kinase C (PKC), calcineurin, gpl30-STAT, insulin-like growth receptor, fibroblast growth factor, and transforming growth For example, binding of the cell surface receptors for PE, and leads to activation of phospholipase C, resulting in the production of diacylglycerol and inositol triphosphate, mobilization of intracellular Ca2+, and activation of protein kinase C. The extent to which these signaling pathways interact during cardiac hypertrophy is unknown (Molkentin et al. (2001) Rev. 63: 391-426).

The protein kinase C (PKC) family of calcium and/or lipid-activated serine-threonine kinases functions downstream of nearly all membrane-associated signal transduction pathways (2001) 63: 391-426). Approximately 12 different isozymes comprise the PKC family, which are broadly classified by their activation characteristics. The conventional PKC isozymes and are calcium-and lipid-activated, while the novel isozymes and and atypical isozymes and) are calcium independent but activated by distinct lipids et al. (2000) J. 279: 247- Once activated, PKC isozymes translocate to discrete subcellular locations through direct interactions with docking proteins termed RACKs (Receptor for Activated C Kinases), which permit specific substrate recognition and subsequent signal transduction (Mochly-Rosen, D (1995) Science 268: 247-251).

Reports have associated PKC activation with hypertrophy, dilated cardiomyopathy, ischemic injury, or mitogen stimulation (DeWindt et al. (2000) J. 275: 13571-13579; Gu & Bishop (1994) Circ. Res. 75: ; Jalili et (1999) A771. J. Physiol. 277: H2298-H2304; Takeishi et al (1999) Physio. 276: H53-H62). For example, hemodynamic pressure overload stimulation in rodents promotes translocation of and In diverse cultured cardiomyocytes, agonists and stress stimuli are also potent activators of PKC isozyme translocation. Isozyme-specific peptide inhibitors have been employed in cultured cardiomyocytes and in transgenic mice to afford greater specificity of PKC inhibition.

Specifically, overexpression of a domain peptide in cardiomyocytes blocked phorbol ester-mediated calcium channel activity (Zhang et al. (1997) Circ. Res. 80: 720-729), while a PKCs inhibitory or activating peptide affected inotropy and ischemia-induced cellular injury (Gray et (1997) Biol. Chem. 272: ; Johnson et al. (1996) J. Biol.

271: 24962-24966; Dorn et al. (1999) Proc. 96: 12798-12803). Additionally, adenovirus-mediated gene transfer of into cultured adult rabbit ventricular myocytes augmented basal myocyte contractility and calcium transients. The results in myocytes suggest that PKC£ functions to enhance cardiac contractile performance (Baudet et al. (2001) Cardiovasc.

Res. 50: 486-494).

Phorbol esters exert acute biologic effects on metazoan cells, mostly consistent with immediate activation of multiple PKC isozymes. In cardiac myocytes, PMA is a potent inducer of many PKC isozymes including, but not limited to, and translocation and activation (Braz et (2002) J. 156: 905-919). Thus acute PMA administration may be used to examine the immediate, but non-specific effects of PKC translocation on alterations in cardiac inotropy and contractility. Acute phorbol ester administration has been used to assess the hypothesis that PKC isozymes regulate, in part, the contractile performance of the whole heart or isolated myocytes. For example, using isolated chicken ventricular myocytes, PMA treatment produced a concentration and time-dependent decrease in the amplitude of cell shortening, reaching a maximum of a decrease at 1 uM drug (Leatherman et al.

(1987) J. : H205-209). Consistent with this effect, PMA produced a decrease in intracellular calcium concentration and the rate of calcium reuptake. In contrast, PMA pretreatment of papillary muscles from the heart potentiated alpha 1-adenoceptor-mediated positive inotropy, demonstrating the non-selective effects of using PMA (Otani et aL (1988) Circ. Res. 62: 8-17). An analysis performed in isolated ventricular myocytes from adult rats showed that PMA has an acute negative contractile effect (Capogrossi et al. (1990) Circ. Res. 66: 1143-1155). These authors used steady field stimulation of adult myocytes at 1 Hz in 1 mM calcium, after which PMA was applied resulting in a decrease in twitch amplitude to approximately 60% of control. In single cardiac myocytes myofilament responsiveness to calcium was not affected by PMA, but that the negative inotropic effect was due to diminished amplitude of the calcium transient. In contrast to this report, a separate study using a slightly different phorbol ester, 13-acetate (TPA), showed significant increases in cell shortening and an increase in the rate of change in cell length during relaxation, suggesting enhanced contractility by activation of PKC isozyme (s) (MacLeod et J.

444: 481-498). A more elaborate study in both isolated cardiac myocytes and whole guinea-pig hearts showed a significant positive inotropic response with M PMA, but a negative inotropic response at concentrations higher than 10'1° M PMA (Ward and Moffat (1992) 24: 937-948).

The results discussed above suggest that phorbol ester-mediated alterations in cardiac contractility are complex.

Transgenic mice have been generated with altered PKC isozyme expression in the heart.

Overexpression of either wild-type or a constitutively active deletion mutant in a mouse heart was reported to induce cardiomyopathy (Wakasaki et al. (1997) Natl. Acad. Sci. USA 94: 9320-9325; Bowman et al (1997) J. Clin. Invest. 100: 2189-2195) but more recent investigation has suggested that lower levels of expression or adult onset activation benefits ischemic recovery (Tiang et (1999) Proc. 96: 13536-13541 ; Huang et al. (2001) Am. J.

: Three groups have also reported transgenic mice with altered PKC8 or PKC6 activity in the heart. Expression of a PKCs or PKC6 activating peptide in the mouse heart was associated with a physiologic activation of each isozyme and a mild hypertrophic response (Mochly-Rosen et al. (2000) (2001) Natl. Acad.

Sci. 98: 11114-11119) Similarly, overexpression of an activated mutant PKC£ in the mouse heart was reported to induce significant cardiac hypertrophy (Takeishi et (2000) Circ.

Res. 86: 1218-1223), but such a result is likely dependent on the absolute levels overexpression and activity (Pass et (2001) J. 280: H946- H955). While a number of studies have demonstrated associations between various PKC isozymes and cardiac hypertrophy or ischemic injury, the necessary and sufficient functions of specific PKC isozymes are still debated. For example, while transgenic overexpression or the mouse heart can initiate cardiac hypertrophy, gene targeting for these 3 isoforms did not overtly affect the heart, nor were null mice defective in their ability to mount a hypertrophic response (Roman et al. (2001) J. 280: H2264- H2270). Collectively these results highlight the confusion in the art as to the PKC isozymes' roles as regulators of cardiac contractility. knock out mice have also been generated by Legites et al. Mol. 16,847-858) showing that enhances insulin signaling through

is the predominant PKC isofonn expressed in the small and large mammal heart, yet little is understood of its function in this organ (Pass et al. ; Ping et (1997) Circ. Res. 81: 404-414). While a number of correlative studies have been published showing associations between activation and cardiac hypertrophy or heart failure, almost no causal or mechanistic data have been reported. Gain-and loss-of function analysis of PKC isozyme function using cultured neonatal cardiac myocytes and recombinant adenoviruses expressing either wild-type or dominant negative mutants of and has been performed. It was reported that regulates the hypertrophic growth of cultured neonatal myocytes in part through ERK1/2, but its role in cardiac contractility is unknown (Braz et (2002) supra).

Similarly, antisense phosphorothioate oligonucleotides against in cultured neonatal cardiac myocytes reduced hypertrophic gene expression following agonist stimulation (Kerkela et al.

(2002) Mol. Pharmacol. 62: 1482-1491). However, none of these observations include a mechanical assessment in vivo.

Little is understood of the role that various PKC isoforms play in potentially regulating cardiac contractility. PKC isoforms are known to directly phosphorylate sarcomeric proteins such as which has been reported to affect the rate of maximal ATPase activity due to actin- myosin interactions (de Tombe & Solaro. (2000) 28: 991-1001). However, it remains unclear if PKC-mediated phosphorylation of contractile proteins significantly alters cardiac performance, in contrast to the well characterized effects

Thus, a mechanistic assessment of PKCa's in vivo role is desirable. It is of importance to develop methods of modulating activity in cardiac tissue. It is also important to develop a model transgenic system for identifying modulating and anti-cardiomyopathic compounds and studying cardiomyopathies.

Treatment of heart failure in humans is based, in part, on the underlying causes, if known, and other factors including the severity of the disease, existing medications and other coinciding risk factors (for example, coronary artery disease, hypertension, valvular defects or hyperlipidemia). Advanced heart failure in patients may consist of both acute and chronic presentations, which may require varying treatments. Thus, current heart failure strategies target either acute decompensated heart failure (ADHF) or the chronic remodeling effects of heart failure. Treatment of ADHF is an unmet medical need, serving as the primary diagnosis for approximately 1 million hospital admissions per year in the United States and is the secondary diagnosis for another 2 million hospitalizations

38: 649-660). ADHF is marked by functional deficits due to acute injury to the heart, e. g., myocardial infarction, arrhythmia, or may be precipitated by complications of chronic heart Prog. Dis. 43: 433-455).

In either case, the patient requires immediate intervention for successful outcomes. Current treatments for ADHF depend largely on symptoms upon presentation to the emergency room, but may include inotropes (such as dobutamine and milrinone), intravenous diuretics (such as furosemide) and/or vasodilators (such as Nesiritide (RTM) in order to improve myocardial performance and maintain sufficient cardiac output (DiDomenico RJ et The goal of treatments using these drugs is to enhance or restore cardiac contraction and relaxation acutely and provide symptomatic improvement. In addition to ADHF, the drugs mentioned above may be administered in any setting of cardiac dysfunction (such as left ventricular dysfunction as a result of sepsis) when it is deemed medically necessary for survival, regardless of the etiology. In chronic heart failure, the drug regime is distinct and commonly includes agents such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics and/or receptor blockers. These drugs are not administered for ADHF and some may in fact be counter-productive in this setting (e. g., receptor blockers). While these drugs provide little or no immediate improvement in cardiac contraction or relaxation, they have been demonstrated to improve survival and cardiac remodeling in heart failure patients (Aronow WS. (2003) Heart Dis. 5: 279-294). Therefore, there is a need to identify novel targets and their modulators to provide sufficient acute and chronic benefits in heart failure.

SUMMARY OF THE INVENTION The inventions are based on the novel discovery that regulates cardiac contractility and cardiomyopathy and therefore both acute decompensated heart failure (ADHF) and chronic heart failure. Accordingly, it is believed that modulation of activity may provide therapeutic means for enhancing cardiac inotropy and ventricular performance. Transgenic animals of the invention are useful in identifying compounds for prophylaxis and treatment of disorders modulated by cardiac contractility, and cardiomyopathy including cardiac hypertrophy.

These animals are also useful for investigative purposes, for examining signal transduction pathways involved in response to hypertrophic signals. The invention also details a process for measuring activation in vivo to screen for pharmacologic modulators of PKCa activity.

Compositions of the invention include transgenic mice, transgenic cells and transgenic tissues. In an embodiment, transgenic mice, cells and tissues of the invention comprise an expression cassette comprising a cardiac tissue-preferred regulatory sequences operably linked to a nucleotide sequence (SEQ ID NO: NCBI Accession No. X04796) or a fragment or variant thereof. A variant is set forth in SEQ ID NO: and encodes a polypeptide (SEQ ID NO: 8) exhibiting a dominant-negative effect. The cell expressing the expression cassette exhibits altered expression or activity. In another aspect, the transgenic mouse of the invention exhibits altered cardiac contractility. In another aspect, a mouse of the invention exhibits altered susceptibility to cardiomyopathy.

In another aspect, transgenic mice, transgenic cells and transgenic tissue comprise at least one disrupted gene. In one aspect, the disruption is sufficient to decrease or eliminate expression levels. In another aspect, a null mouse of the invention exhibits altered cardiac contractility. In another aspect, a mouse of the invention exhibits an altered susceptibility to cardiomyopathy.

In one aspect, methods of identifying compounds that modulate cardiac contractility are disclosed, comprising: providing a first and a second cell, tissue, or mouse, expressing a gene; administering a compound of interest to said first cell; incubating both the first and second cells for a suitable, predefined period of time; measuring the activity of in said first and said second cell; and identifying those compounds that modulate the activity of in said first cell compared to activity in said second cell as modulators of cardiac contractility.

In another aspect, methods of identifying compounds that modulate cardiomyopathy are disclosed, comprising: providing a first and a second cell expressing protein; administering a compound of interest

to said first cell; incubating both the first and second cells for a suitable, predefined period of time; measuring the activity of in said first and said second cell; and identifying those compounds that modulate the activity of in said first cell compared to activity in said second cell as modulators of cardiomyopathy.

In another aspect, methods of identifying compounds that modulate activity are disclosed, comprising: providing a first and a second cell expressing protein ; administering a compound of interest to said first cell; incubating both the first and second cells for a suitable, predefined period of time; measuring the activity of in said first and said second cell; and identifying those compounds that modulate the activity of in said first cell compared to activity in said second cell as modulators of activity.

For the above-described assays to identify compounds that modulate cardiac contractility, cardiomyopathy, and activity; any cell expressing suitable levels of protein could be primary cells, or tissues. Cells, and tissues from transgenic, or knock out mice; the transgenic animals themselves ; and the dominant negative mutants of the invention are suitable for the purpose.

Modulators of activity include inhibitors or activators of the various activities, including, but not limited to, the enzymatic activity; the translocation activity and the binding to various RACKs.

In another aspect, the compounds identified using above-described methods could be further validated using assays that utilize various cell culture, cultured tissues, or animal models of cardiac contractility, or cardiomyopathy as described herein.

In another embodiment, the invention provides a method of preferentially modulating activity in cardiac tissue. The method comprises providing a transgenic mouse comprising a stably incorporated expression cassette in the genome of at least one cell. The stably incorporated expression cassette comprises a cardiac preferred regulatory sequence operably, linked to the nucleotide sequence set forth in SEQ ID NO: or fragment or variant thereof.

Variants of interest include, but are not limited to, dominant negative mutations such as the site directed mutant having the nucleotide sequence set forth in SEQ ID NO: 7. The invention further comprises determining the expression levels in the cardiac tissue of the mouse. In an aspect of the method, the mouse exhibits altered cardiac contractility. In another aspect, the mouse exhibits an altered susceptibility to cardiomyopathy.

In an embodiment, the invention provides a method of modulating expression in a mouse. The method comprises providing a transgenic mouse comprising at least one disrupted gene in the genome of at least one cell. The invention further comprises determining the PKCa expression levels in the mouse.

In an embodiment, the invention provides a method of treating or preventing an acute heart failure resulting from abnormal cardiac contractility in an animal. The method comprises the step of administering a modulating compound to the animal. In an aspect of the invention, the modulating compound is administered to the animal's cardiac tissue. In an aspect of the invention, the modulating compound is a inhibitor. In an aspect of the invention, the method increases the animal's cardiac contractility. Suitable animals include, but are not limited to, mice, guinea pigs, hamsters, humans, rabbits, dogs, pigs, goats, cows, rats, monkeys, chimpanzees, sheep, and zebrafish.

In an additional embodiment, the invention provides a method of treating or preventing a cardiomyopathy in an animal. The method comprises the step of administering a modulating compound to the animal. In an aspect of the invention, the modulating compound is administered to the animal's

cardiac tissue. In an aspect of the invention, the modulating compound is a inhibitor or agonist. In an aspect of the invention, the method decreases the animal's susceptibility to cardiomyopathy. Suitable animals include, but are not limited to, mice, guinea pigs, hamsters, humans, rabbits, dogs, pigs, goats, cows, rats, monkeys, chimpanzees, sheep, and zebrafish.

inhibitors that may be used in the treatment of cardiac contractility or cardiomyopathy include, but are not limited to, nucleic acids, antibodies, small molecules, activator and inhibitor peptides, and Ro-32-0432, LY333531 and Ro-31-8220.

The invention also provides kits for performing a method of identifying a modulating compound. In an aspect of the invention a kit for identifying a modulating compound comprises a indicator polypeptide. In an aspect of the invention a kit for identifying a modulating compound comprises a cell comprising a indicator polypeptide.

DESCRIPTION OF SEQUENCE LISTING Name Species SEQ ID NO: Genbank Accession DNA Protein Number PKCa 07yctolagus cu7ticulus 1 2 X04796 PKCa Mus musculus 3 4 X52685 PKCa Honao sapiens 5 6 X52479 PKCa dominant Oryctolagus cutziculus 7 8 negative mutant Deleted exon Mus nzusculus 9 10 5'Long primer Mus musculus 11 5'short primer Mus musculus 12 3'Longprimer Mus mllsculus 13 3'short primer Mus musculus 14 a MHC promoter Mus musculus 15 U71441 sequence BRIEF DESCRIPTION OF THE DRAWINGS Figure 1 presents generation and characterization of the murine gene disruption transgenic mice. Details of the experiments are described elsewhere herein. Panel A depicts a schematic of the murine genomic locus and the targeting vector used to replace the ATP binding exon (E) with a neomycin resistance gene (neo). The approximate locations of SalI, EcoRV, and ClaI restriction enzyme sites are indicated. The approximate location of the nucleotide sequence used as a genomic probe to identify transgenic mice is also indicated. SEQ ID NO: 9 and 10 provide nucleotide and amino acid sequence of the exon deleted and SEQ ID NOs: 11-14 are the primers used to create the PCR products. Panel B depicts results of a Southern blot assay of embryonic stem cells. Lane 1 contains DNA from a wildtype cell, and Lane 2 contains DNA from a transgenic cell. Panel C depicts results of Western blot analysis of in protein preparations from hearts of wild-type, heterozygous +/-, and PKCa-/- transgenic mice. Proteins from wildtype mice are presented in lanes 1-2; proteins from PKCa+/- mice are presented in lanes 3-4 ; and proteins from presented in lanes 5-6.

Figure 2 depicts results of Western blot analysis and PKC£ in protein preparations from hearts of wildtype and Proteins from wildtype mice are presented in lanes 1-4; proteins from are presented in lanes 5-8. Proteins in lanes 2,5, and 6 were obtained from the hearts of animals that underwent a sham procedure. Proteins in lanes 3,4, 7, and 8 were obtained from the hearts of animals that underwent transverse aortic constriction (TAC). The proteins were separated into soluble (S) (Lanes 1,3, 5, and 7) and particulate (P) (Lanes 2,4, 6, and 8) fractions prior to polyacrylamide gel electrophoresis.

Figure 3 presents results obtained from invasive hemodynamic assessment of ventricular performance as maximal dP/dt in anesthetized close-chested mice at baseline or in response to increasing amounts of dobutamine infusion Maximal increments are in The dobutamine dose is indicated as ng dobutamine/g mouse/min. Results obtained from wildtype mice are indicated with circles (N=6). Results obtained from are indicated with triangles (N=6). Experimental details are described elsewhere herein.

Figure 4 presents results of an analysis of cardiac ventricular performance in wild type (Wt) and homozygous deletion (PKCa-/-) mice. Results obtained from wildtype mice are indicated with solid bars. Results obtained from are indicated with cross-hatched bars. In each panel, the first two bars indicate data obtained from 2 month old mice, and the last two bars indicate data obtained from 10 month old mice. (N=4 mice in each group). Panel A presents maximal dP/dt obtained from ex vivo

working hearts. Panel B presents the left ventricular pressure (LVP) as measured in mmHg.

Figure 5 presents results obtained from wildtype (NTG, circles) and deletion null, triangles) mice. Panel A presents the heart rate (HR) in beats per minute (bpm) in response to increasing dobutamine. Panel B presents the mean arterial pressure (MAP) in mmHg in response to increasing dobutamine. (Heart rate and mean arterial pressure were also described elsewhere herein.

Figure 6 presents generation and characterization of transgenic mice.

Experimental details are described elsewhere herein. Panel A depicts a schematic of the cardiac tissue-preferred a-myosin heavy chain (a-MHC) promoter (Genbank U71441; SEQ ID NO: 15) operably linked to the rabbit gene (SEQ ID NO: 1). Panel B depicts results of Western blot analysis of PKC isoforms in protein preparations from hearts of wildtype and transgenic mice. The isoform of interest and is indicated on the left hand side of the blots. Lanes 1 and 2 contain proteins from wildtype (NTG) mice; lanes 3 and 4 contain proteins from transgenic mice TG). The transgenic mice are also referred to as overexpressing mice. Panel C depicts results of Western blot analysis with antibodies to the site. Proteins were obtained from hearts of non-transgenic mice (lanes 1 and 2); (lanes 3 and 4), and overexpressing transgenic mice (lanes 5 and 6).

Figure 7 presents results of cardiac ventricular performance assessments. Results obtained from wildtype mice are indicated with a white bar; results obtained from transgenic mice are indicated with a solid bar. Panel A presents the results of an assessment of fractional shortening percentage by echocardiography. Panel B presents results obtained from analysis of ventricular performance by isolated working hearts as maximal dP/dt (Maximum dP/dt).

Figure 8 depicts results obtained from heart-weight (HW) to body-weight (BW) ratio analysis of cardiac hypertrophy in unstimulated male transgenic mice, Four mice were assessed at each time point (2,4, 6, and 8 months).

Figure 9 presents results of a peak shortening assay performed on wildtype adult rat myocytes. The cells were infected with adenoviruses encoding p-galactosidase white bar), wildtype (solid bar), and dominant negative (dn-PKCa, striped bar). The number of cells analyzed is shown below each bar.

Figure 10 presents results from a series of assays involving alterations in activity and phospholamban phosphorylation status. Details of the experiments are described elsewhere herein. Panel A depicts results of a Western blot of protein from three wildtype (Wt, Lanes 4-6) and three (Lanes 7-9) hearts at two months of age probed with antibodies to SERCA2, calsequestrin (CSQ), and phospholamban (PLB). Lanes 1-3 (Standard) were loaded with the indicated amount of protein. The relative quantitation of total phospholamban (PLB) versus SERCA2 is also presented (panel C). Data from wildtype hearts is indicated with solid bars; data from is indicated with white bars. Panel B depicts the quantitation results of a Western blot of protein from three wildtype (Wt, Lanes 4-6) and three (Lanes 7-9) hearts probed with PLB serine 16 phospho-specific antibody. Lanes 1-3 (Standard) were loaded with the indicated amount of protein. The relative quantitation of total PLB (PLB tot) versus phosphorylated PLB (phos-PLB) is also presented (panel D). Data from wildtype hearts is indicated with solid bars; data from is indicated with white bars.

Figure 11 depicts the results of a Western blot of protein from wildtype adult rat ventricular myocytes infected with adenoviruses encoding p-galactosidase or adenoviruses encoding dominant negative (AdPKCa-dn) for the indicated days. The blot was probed with PLB serine 16 phospho-specific antibody.

Figure 12, panel A presents the results of RNA dot blot analysis of wildtype (Wt) and (Null) mice at the indicated ages. The dot blots were probed with phospholamban (PLB), SERCA2, and GAPDH specific probes. Panel B presents the results analysis of two wild-type and two PKCa-/- (Null) mice. The number of cycles performed is indicated.

Primers specific to PLB, SERCA2a, and ribosomal protein L7 (L7) were used.

Figure 13 presents results from a series of assays involving alterations in levels and phospholamban phosphorylation status. Panel A depicts results of a Western blot of protein from three wildtype (Wt) and three transgenic TG) hearts at two months of age probed with antibodies to SERCA2, calsequestrin (CSQ), and phospholamban (PLB). The relative quantification of total phospholamban (PLB) versus SERCA2a is presented in Panel B. Data from wildtype hearts is indicated with solid bars; data from TG hearts is indicated with white bars. Panel C depicts the results of a Western blot of protein from three wildtype (Wt) and three transgenic TG) hearts probed with PLB serine 16 phospho-specific antibody.

The first three lanes (Standard) were loaded with the indicated amount of protein. The relative quantification of total PLB (PLB tot) versus phosphorylated PLB (phos-PLB) is also presented in Panel D. Data from wildtype hearts is indicated with solid bars; data from TG hearts is indicated with white bars.

Figure 14 presents the results of a series of assays assessing the calcium transient in PKCa-/-cardiac myocytes. Details of the experiments are described elsewhere herein. Panel A depicts representative Fura-2 emission tracing of calcium transients from an adult wildtype and cardiomyocyte (2 months of age). Panel B presents the peak calcium release (left) and 80% of relaxation time right) measured in seconds. Results from myocytes from wildtype mice are indicated by white bars. Results from myocytes from PKCa-/- mice are indicated by solid bars.

Figure 15, panel A presents representative Indo-1 AM emission tracings from wild-type (wt) and PKCa-/- (KO) myocytes prior to and subsequent to caffeine administration. The point of caffeine stimulation is indicated. Panel B presents results obtained from assessment of caffeine induced Ca2+ transients in myocytes. Wild-type myocytes (WT) are indicated with a white bar (n myocytes are indicated with a solid bar (n = 37).

Figure 16 presents traces of mean peak calcium density obtained at depolarizing voltage steps from-50 mV to +40 mV in 10 mV increments. Results from wildtype (NTG) cells are in the left trace; results from (PKCa-KO) are in the right trace.

Figure 17 depicts the results of total phosphatase, specific, and PP2A specific enzymatic assays performed on wild-type (Wt, solid bars) and empty bars).

Figure 18 depicts the result of and PP2A-specific enzymatic assays from wildtype (Wt, solid bars) or transgenic (overexpressing) hearts a-TG, empty bars). N=3 separate assays from 3 hearts each.

Figure 19 presents the results of PP2A-specific enzymatic assays from neonatal cardiomyocytes acutely infected with the indicated adenoviruses: adenovirus encoding galactosidase white overexpressing adenovirus (AdPKCa wt, solid bars); and dominant negative adenovirus dn, striped bars). Phosphatase activity is presented as counts per minute (cpms) per protein.

Figure 20, panel A presents an SDS-PAGE of E. coli purified Inhibitor-1 wildtype protein subjected to phosphorylation with and purified protein kinase C. Each lane contains an aliquot from the indicated

time point (10,30, or 60 minutes). The results are summarized in the graph below the gel. The graph depicts the amount protein at the indicated time points. Panel B presents an of E. coli purified 1-1 wild-type (Wt) or S67A mutant protein subjected to phosphorylation with and purified protein kinase C.

The graph below the gel indicates the relative amount wild-type or S67A protein.

Figure panel A presents a Western blot of extracts from adenoviral-infected neonatal cardiomyocyte cultures incubated with antisera to Inhibitor-1 (I-1). Extracts were prepared from cultures infected with adenovirus expressing wild- type wt), dominant negative mutant dn), Inhibitor-1 and P-galactosidase (I-1 + Inhibitor-1 and wild-type + wt), and Inhibitor-I and dominant negative + dn). The extracts were immunoprecipitated with The immunoprecipitants were resuspended, electrophoresed, transferred to a membrane, and hybridized with antisera. A membrane strip containing the protein band was hybridized with PP I c antisera (shown below the I-1 treated Western blot). The hybridized proteins were quantified and the results summarized in Panel B. Panel B depicts the relative amounts of I-1 precipitated from each extract: Inhibitor-1 and + solid bar), Inhibitor-1 and wild-type + Ad wt), empty bar), and Inhibitor-1 and dominant negative + dn, striped bar).

Figure 22 presents a Western blot with I-1 phospho-specific antibodies against threonine- 35 and serine-67 from neonatal cardiomyocyte cultures.

Figure 23 presents quantification of independent Western blots for I-1 phospho-serine 67 from wildtype, PKCa-l-and PKCa transgenic hearts. Typical Western blots are shown beneath the graph.

Figure 24, Panel A presents a quantification of western blotting for total PKC a protein levels in"normal"human donor hearts (empty bars, Donor) or dilated cardiomyopathic hearts (solid bars, HF) in failure. Panel B presents western blot quantification between and phosphorylation in"normal"donor hearts (empty bars, Donor) and failing hearts (solid bars, HF).

Figure 25 presents micrographs of protein localization in adult rat cardiac myocytes at baseline or after PMA + PMA) stimulation.

Figure 26 depicts the results of an assessment of heart function and in wildtype (Wt) and twelve weeks after either a TAC procedure or sham operation. Results obtained from wildtype, sham-operated mice are indicated with empty bars; results obtained from sham-operated mice are indicated with solid bars, results obtained from wildtype, TAC mice are indicated with a cross-hatched bar, and results obtained from TAC mice are indicated with a striped bar. The left side of the graph presents results of ex vivo working heart preparations (Maximum dP/dt measured in mmHg/sec). The right side of the graph presents the left ventricular pressure (LVP) in mmHg.

Figure 27 depicts the results of an assessment of heart function and hypertrophy in wildtype (Wt) and PKCa-l mice twelve weeks after either a transverse aortic constriction (TAC) procedure or sham operation. Results obtained from wildtype, sham-operated mice are indicated with empty bars; results obtained from sham-operated mice are indicated with solid bars, results obtained from wildtype, TAC mice are indicated with a cross-hatched bar, and results obtained from TAC mice are indicated with a striped bar. Panel A presents the left ventricular end diastolic (LVED) and left ventricular end systolic (LVES) dimensions in mm.

Panel B presents results of echocardiography analysis of fractional shortening (FS).

Figure 28 depicts the results of an assessment of heart function, hypertrophy, and gross heart

morphology in wildtype (Wt), and Results obtained from wildtype mice are indicated with white bars; results obtained from PKCa-l mice are indicated with solid bars, results obtained from are indicated with a hatched bar, and results obtained from are indicated with a striped bar. Panel A presents the left ventricular end diastolic (LVED) and left ventricular end systolic (LVES) dimensions in mm.

Panel B presents results of echocardiography analysis of fractional shortening (FS).

Figure 29 depicts the results of an assessment of heart function, hypertrophy, and gross heart morphology in wildtype (Wt), and Results obtained from wildtype mice are indicated with white bars; results obtained from are indicated with solid bars, and results obtained from are indicated with a striped bar. The left side of the graph presents results of ex vivo working heart preparations (Maximum dP/dt measured in The right side of the graph presents the left ventricular pressure (LVP) in mmHg.

Figure 30 presents heart weight (HW) to body weight (BW) ratios (N=4 for each group).

Results obtained from wildtype mice are indicated with white bars; results obtained from PKCa-/- mice are indicated with solid bars, results obtained from are indicated with a hatched bar, and results obtained from are indicated with a striped bar.

Figure 31 presents gross heart morphology assessed by Hematoxylin and Eosin staining of heart histological sections in wildtype (Wt), and MLP-/-PKCa-l-mice.

Figure 32 presents results phosphatase assays from the hearts of adult wildtype (Wt, empty bar), bar), PPlc transgenic (solid bar), and PKCa l x PP 1 c (striped bar) mice (N=4 mice in each group).

Figure 33 presents echocardiographic assessment of fractional shortening (FS) from the indicated groups of mice (N=4 each): Wildtype (Wt, empty bar) ; PPlc transgenic solid bar); and PKCa-/-X striped bar).

Figure 34 presents ex vivo working heart assessment of ventricular performance in wildtype (wt, white bar; PPlc black bar) and PKCa-/-X striped bar).

Panel A presents the maximum dP/dt. Panel B presents the minimum Panel C presents the left ventricular pressure (LVP) in mmHg.

Figure 35 presents an analysis of mortality in two heart failure models. Panel A presents percent survival of wild-type (Wt, empty bars) and PKCa-/- (PKCa-/-, solid bars) at the indicated time points after a TAC operation. Panel B presents percent survival of wild-type (Wt, empty bars), PKCa-/- (PKCa-/-, solid bars), MLP-/- (MLP-/-, hatched bars), and (Double, striped bars) mice at the indicated ages.

Figure 36 presents maximum (Panel A) and minimum (Panel B) dP/dt values obtained from isolated hearts infused with phorbol myristate acetate (PMA). Results obtained from wild- type hearts are indicated with empty circles; results obtained from PKCa-/-hearts are indicated with solid circles. Four hearts were analyzed in each group, and the error bars represent standard error of the mean. The PMA dosages are indicated.

Figure 37 presents results of a Western blot analysis of the indicated PKC isoforms and in the normal human heart. The isozymes are bracketed. In Panel A the left three lanes contain recombinant protein standards generated in bacteria (standard). The right six lanes contain proteins from six normal human hearts (Human heart samples). Panel B presents a quantification of the amounts of each isozyme relative to the total protein content of the samples. The amount of each isozyme is indicated in ng/50 of total

lysate. The PKC of interest is indicated below each bar. The error bars represent the standard error of the mean.

Figure 38 presents results obtained from an assessment of acute cardiac contractility in ex vivo working heart preparations. Results obtained from the control group of mice are indicated with empty bars; results obtained from the Ro-32-0432 treated mice are indicated with solid bars.

Baseline results are indicated. The data obtained upon infusion of Ro-32-0432 or the vehicle control are indicated (Infusion). Values throughout the concentration time course (7 minutes per 10 different incremental concentrations) were summated for statistical purposes, representing an average dosage of approximately 1 X M. Only the Ro-32-0432-infused group showed a statistically significant increase (p <0.05).

Figure 39 presents micrographs of indicator polypeptide in cultured cells treated with DMSO (PKCa-GFP + vehicle) or with PMA + PMA 60 minutes).

Figure 40 presents results obtained from an assessment of acute cardiac inotropic and lusitropic function after infusion of LY333531 at the indicated doses, represented by maximum dP/dt (Figure 40A) and minimum dP/dt (Figure 40B), respectively, in vivo in normal Sprague- Dawley and Lewis rats.

Figure 41 shows the percent increase in maximum dP/dt from baseline (B/L) following infusion of Ro-31-8220 in a rat model of myocardial infarction.

DETAILED DESCRIPTION OF THE INVENTION The present invention provides for modulation of cardiac contractility in acute heart failure, and cardiomyopathy in heart failure in general. Compositions of the invention include transgenic animals comprising either a nucleotide sequence or animals with a disruption in nucleotide sequence. The invention further comprises cells and tissues isolated from these mice. The invention provides methods of modulating activity, expression levels, cardiac contractility, and susceptibility to cardiomyopathies, and acute heart failure. The invention provides kits for performing the methods of identifying modulating compounds.

The invention relates to compositions and methods drawn to gene (SEQ ID NO:

In an embodiment, an animal is stably transformed with an expression cassette comprising a cardiac-preferred regulatory sequences operably linked to a nucleotide sequence. In an embodiment, an animal of the invention is stably transformed with an expression cassette comprising a cardiac-preferred regulatory sequences operably linked to a fragment or variant of the nucleotide sequence such as the dominant negative variant set forth in SEQ ID NO : 7.

In another embodiment, an animal of the invention is stably transformed with an isolated nucleic acid molecule that disrupts the native nucleotide sequence such that expression levels are decreased. In one aspect, the cardiac-preferred regulatory sequences are cardiac- preferred promoter sequences.

In an embodiment, the genome of a germ-line cell of a transgenic animal comprises the nucleotide sequence of interest. A transgenic cell is a cell isolated from a transgenic animal of the invention comprising at least one expression cassette or disruption cassette. Transgenic tissue, e. g. cardiac tissue, is tissue comprising transgenic cells.

In embodiments involving disruption cassettes, the nucleotide sequence of interest may be flanked by nucleotide sequences that naturally occur in the genomic DNA of the cell into which the nucleic acid

molecule is transformed.

Fragments and variants of the nucleotide sequence and protein encoded thereby are also encompassed by the present invention. intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence exhibit a activity. Alternatively, fragments of a nucleotide sequence are useful as hybridization probes. A biologically active portion of a can be prepared by isolating a portion of one of the nucleotide sequences of the invention, and assessing the activity of the encoded portion of the protein.

One of skill in the art would also recognize that genes and proteins from a species other than those listed in the sequence listing, particularly mammalian species, would be useful in the present invention. One of skill in the art would further recognize that by using probes from the known species'sequences, or genomic sequences homologous to the known sequence could be obtained from the same or alternate species by known cloning methods. Such homologs and orthologs are included in the definition gene and proteins of the invention.

Thus, a fragment of a protein kinase C-a nucleotide sequence may encode a biologically active portion of a protein kinase C-a or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a can be prepared by isolating a portion of one of the nucleotide sequences of the in vitro), and assessing the activity of the encoded portion of the protein. Nucleic acid molecules that are fragments of a protein kinase C-a nucleotide sequence comprise at least 16,20, 50,75, 100,150, 200,250, 300,350, 400,450, 500,550, 600,650, 700,800, 900,1, 000,1, 100, 1,200, 1,300, 1400,1450, 1500,1550, 1600,1650, 1700,1750, 1800,1850, 1900, 1950, 2000, 2050,2100, 2150,2200, 2250,2300, 2350,2400, 2450,2500, 2550,2600, 2650,2700, 2750, 2800,2850, 2900,2950, 3000,3050, 3100,3150, 3200,3250, 3300,3350, 3400,3450, 3500, or 3524 nucleotides, or up to the number of nucleotides present in a full-length protein kinase C-a nucleotide sequence disclosed herein, or that contain additional sequences from the genomic locus alone or in combination with the sequence discussed above..

By"variants"are intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides of the invention. Naturally occurring variants such as these can be identified with the use of known molecular biology techniques, as, for example, with polymerase chain reaction and hybridization techniques that are known in the art. In order to isolate orthologs and other variants generally stringent hybridization conditions are utilized mainly dictated by specific sequence, sequence length, GC content and other parameters. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis.

Variants may also contain additional sequences from the genomic locus alone or in combination with other sequences.

Variant proteins may be derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids; deletion or addition of one or more amino acids; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention may or may not retain biological activity. Such variants may result from, for example, genetic polymorphism or from human manipulation. An exemplary variant protein is encoded by the nucleotide sequence set forth in SEQ ID NO: 7. The variant protein encoded by the nucleotide sequence set forth in SEQ ID NO: 7 exhibits dominant negative effects.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. For example, amino acid sequence variants of the proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are known in the art. See, for example, Kunkel (1985) Proc.

Acad. : 488-492; Kunkel et aL Methods in : 367-382; U. S. Patent No. 4, 192; Walker and Gaastra, eds. (1983) Techniques in Molecular Biology (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model (Natl.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences can be manipulated to create a new possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo.

For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the gene of the invention and other known genes to obtain a new gene coding for a protein with an altered property of interest e. g. a dominant negative mutation (Ohba et (1998) Mol. Cell. Biol. 18: 51199-51207, Matsumoto et al. 276: 14400- 14406). Strategies for such DNA shuffling are known in the art.

The"percentage of sequence identity"or"sequence identity"is determined by comparing two optimally aligned sequences or subsequences over a comparison window or span, wherein the portion of the sequence in the comparison window may optionally comprise additions or deletions (i. e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by amino acid residue) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

Percentage sequence identity can be calculated by the local homology algorithm of Smith & Math. 2: 482-485 ; or by the homology alignment algorithm of Needleman & Wunsch, MoL Biol. : 443-445 (1970); either manually or by computerized implementations of these algorithms (GAP & BESTFIT in the GCG Wisconsin Software Package, Genetics Computer Group).

A preferred method for determining homology or sequence identity is by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin et (1990) Natl. Acad. Sci. USA 87,2264-2268 and Altschul, (1993) J. Mol. Evol. 36,290-300), which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. The search parameters for histogram, descriptions, database sequences), cutoff, matrix and filter are generally set at the default-scoring matrix BLOSUM62 for blastp, tblastn, and tblastx (Henikoff et al. (1992) Proc. Natl. Acad. Sci.

USA 89,10915-10919).

As described herein, PKCa genes and proteins, their allelic and other variants (e. g. splice variants), their homologs and orthologs from other species and various fragments and mutants will exhibit sequence variations. Typically, these sequences may exhibit at least about sequence identity, preferably at least about 80% sequence identity, more preferably at least about sequence identity and more preferably at least about 95% sequence identity to the genes and proteins of the invention.

The sequences of the invention are provided in expression cassettes for expression in the animal of interest. The cassette will include 5'and 3'regulatory sequences operably linked to a sequence of the invention. By"operably linked"is intended the transcription and translation of the heterologous nucleotide sequence is under the influence of the regulatory sequences. In this manner, the nucleotide sequences for the nucleotide sequences of the invention may be provided in expression cassettes along with cardiac tissue-preferred promoters for expression in the animal of interest, more particularly in the heart of the animal.

Such an expression cassette is provided with at least one restriction site for insertion of the nucleotide sequence to be under the regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-to-3'direction of transcription, a transcriptional and translational initiation region, and a heterologous nucleotide sequence of interest. In addition to containing sites for transcription initiation and control, expression cassettes can also contain sequences necessary for transcription termination and, in the transcribed region a ribosome-binding site for translation. Other regulatory control elements for expression include initiation and termination codons as well as polyadenylation signals. The person of ordinary skill in the art would be aware of the numerous regulatory sequences that are useful in expression vectors. Such regulatory sequences are described, for example, in Sambrook et Cold Spring Harbor, N. Y.).

The expression cassette comprising the sequence of the present invention operably linked to a promoter nucleotide sequence may also contain at least one additional nucleotide sequence for a gene to be into the organism. Alternatively, the additional sequence (s) can be provided on another expression cassette.

The regulatory sequences to which the polynucleotides described herein can be operably linked include promoters for directing transcription. These include, but are not limited to, the left promoter from bacteriophage the lac, TRP, and TAC promoters from E. coli, the early and late promoters from SV40, the CMV immediate early promoter, the adenovirus early and late promoters, and retrovirus long-terminal repeats.

It is recognized that a nucleotide sequence of the invention can be operably linked to any cardiac tissue preferred promoter and expressed in cardiac tissue. tissue"is intended any tissue obtained from the heart, including but not limited to, tissues developmentally related to the heart such as the pulmonary myocardium.

It is recognized that to increase transcription levels or to alter tissue specificity, enhancers tissue-preference elements may be utilized in combination with the promoter. For example, quantitative or tissue specificity upstream elements from other cardiac-preferred promoters may be combined with the promoter region used to generate the overexpressing mice to augment cardiac-preferred transcription. Such elements have been characterized, for example, the murine TIMP-4 promoter, A and B-type natriuretic peptide promoters, human cardiac troponin I promoter, mouse S100A1 promoter, salmon cardiac peptide promoter, GATA response element, inducible cardiac preferred promoters, rabbit promoter, and mouse a-myosin heavy chain promoter et al. (2002) Acta 1577: 45-52; (1997) Biol.

Chenu. 272: 7464-7472; LaPointe et al (1996) Hypertension 27: 715-722; (1994) Mol. 14: 3115-29; Dellow,

(2001) Res. 50: 3-6; Kiewitz, et (2000) : 207-19; Majalahti-Palviainen, et al (2000) : 731-740; Charron et al. (1999) Molecular Cellular Biology 19: 4355-4365; Genbank U71441; U. S. Provisional Patent Application No: 60/393,525 and 60/454,947 ; and U. S.

A variety of cardiac tissue preferred promoter elements have been described in the literature and can be used in the present invention. These include, but are not limited to, tissue preferred elements from the following species: myosin light chain-2, a-myosin heavy chain, cardiac troponin C, and cardiac a-actin. See, e. g. Franz et al (1997) 35: 560-566; Robbins et al. (1995) 752: 492-505; Linn et al. (1995) Circ. Res. 76: 584- 591; et (1994) Mol Cell Biol. 14 : 1870-1885 ; Hunter et al. (1993) Hypertension 22: 608-617; and Sartorelli (1992) Proc. Natl. Acad. Sci. USA 89:

In other embodiments, the coding region is operably linked to an inducible regulatory element or elements. A variety of inducible promoter systems has been described in the literature and can be used in the present invention. A known and useful conditional system is the binary, tetracycline-based system, which has been used in both cells and animals to reversibly induce expression by the addition or removal of tetracycline or its analogues. Another example of such a binary system is the recombinase system of bacteriophage P 1. For a description of the

Another class of promoter elements are those which activate transcription of an operably linked nucleotide sequence of interest in response to hypoxic conditions. These include promoter elements regulated at least in part by hypoxia inducible Hypoxia response elements include, but are not limited to, the hypoxia response enhancer element the muscle pyruvate kinase HRE; the HRE; and endothelin-1 HRE element, and chimeric nucleotide sequence comprising these sequences. See Bunn and Poynton (1996) Rev. 76: 839-885; Dachs and Stratford (1996) J. Cancer 74 : S126-S132 ; Guillemon and Krasnow (1997) Cell 89 : 9-12; Firth et (1994) Proc. Natl. Acad. Sci. 91: 6496-6500; Jiang (1997) 57: 5328-5335; U. S. Patent No. 5,834, 306).

In addition to control regions that promote transcription, expression vectors may also include regions that modulate transcription, such as repressor binding sites and enhancers.

Examples include the SV40 enhancer, the cytomegalovirus immediate early enhancer, polyoma enhancer, adenovirus enhancers, and retrovirus LTR enhancers.

Where appropriate, the nucleotide sequence of the present invention and any additional nucleotide sequence (s) may be optimized for increased expression in the transformed animal. That is, these nucleotide sequences can be synthesized using species preferred codons for improved expression, such as mouse-preferred codons for improved expression in mice. Methods are available in the art for synthesizing species-preferred nucleotide sequences. See, for example, Wada et (1992) 20 (Suppl.), 2111-2118; Butkus et (1998) Exp 25: S28-33 ; and Sambrook et al. (1989) Molecular Laboratory Manual 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon- intron splice site signals, transposon-like repeats, and other well-characterized sequences that may be deleterious to gene expression. The G-C content of the heterologous nucleotide sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary structures.

In those instances where it is desirable to have the expressed product of the heterologous nucleotide sequence directed to a particular organelle, particularly the mitochondria, the nucleus, the reticulum, or the Golgi apparatus; or secreted at the cell's surface or extracellularly ; the expression cassette may further comprise a coding sequence for a transit peptide. Such transit peptides are known in the art and include, but are not limited to, the transit peptide for the acyl carrier protein, the small subunit of RUBISCO, and the like.

Disruption cassettes are used to interrupt and/or remove a sequence of interest from the "targeting vector"and"disruption cassette"is intended an isolated nucleic acid molecule comprising a 5'flanking region, a disruption region, and a 3'flanking region. Disruption cassettes and methods of their use are known in the art. See Doetschman et al. (1987) Nature 330: 576-578; et (1988) Proc. Natl. Acad. Sci 85: 8583-87; Schwartz et al (1991) Proc. Natl. Acad. Sci. 88: 10416-20; Oliver et al (1997) Proc. Natl. Acad. Sci. 94: 14730-14735; Nagy et al Ed. (2003) Manipulating the Mouse Embryo Cold Spring Harbor Press, Cold Spring Harbor, NY.

Reporter genes or selectable marker genes may be included in the expression cassettes.

Examples of suitable reporter genes known in the art can be found in, for example, Ausubel et

(2002) Current Protocols Molecular Biology. John Wiley & Sons, New York, NY. Selectable marker genes for selection of transformed cells or tissues can include genes that confer antibiotic resistance. Other genes that could serve utility in the recovery of transgenic events but might not be required in the final product would include, but are not limited to, examples such as GUS glucuronidase), fluorescence proteins (e. g. GFP), CAT; and luciferase.

Delivery vehicles suitable for incorporation of a polynucleotide for introduction into a host cell include, but are not limited to, viral vectors and non-viral vectors (Verma and Somia (1997) Nature 389 : 239-242).

A variety of non-viral vehicles for delivery of a polynucleotide are known in the art and are encompassed in the present invention. An isolated nucleic acid molecule can be delivered to a cell as naked DNA (WO Alternatively, a polynucleotide can be delivered to a cell associated in a variety of ways with a variety of substances (forms of delivery) including, but not limited to, cationic lipids; biocompatible polymers, including natural and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; protein transduction domains, and bacteria. A delivery vehicle can be a microparticle.

Mixtures or conjugates of these various substances can also be used as delivery vehicles. A polynucleotide can be associated non-covalently or covalently with these forms of delivery.

Viral vectors include, but are not limited to, DNA viral vectors such as those based on adenoviruses, herpes simplex virus, poxvirus such as vaccinia virus, and parvoviruses, including adeno-associated virus; and RNA viral vectors, including but not limited to, the retroviral vectors.

Retroviral vectors include murine leukemia virus, and lentiviruses such as human immunodeficiency virus. See Naldini et (1996) Science 272: 263-267.

Non-viral delivery vehicles comprising a polynucleotide can be introduced into host cells and/or target cells by any suitable method known in the art, such as transfection by the calcium phosphate coprecipitation technique; electroporation; ; liposome-mediated transfection; ballistic transfection ;

biolistic processes including microparticle bombardment, jet injection, and needle and syringe injection, or by microinjection. Numerous methods of transfection are known to the skilled artisan.

Viral delivery vectors can be introduced into cells by infection. Alternatively, viral vectors can be incorporated into any of the non-viral delivery vectors described above for delivery into cells. For example, viral vectors can be mixed with cationic lipids (Hodgson and (1996) Nature Biotechnol. 14: 339-342); or lamellar liposomes (Wilson et al. (1977) Proe. Natl.

Acad. Sci. 74: 3471-3475 ; 49: 269-272).

For in vivo delivery, the vector can be introduced into an individual or organism by any method known to the skilled artisan.

Any of the regulatory or other sequences useful in expression vectors can form part of the transgenic sequence. This includes intronic sequences and polyadenylation signals, if not already included. In one embodiment, the animal cell can be a fertilized oocyte or embryonic stem cell that can be used to produce a transgenic animal comprising at least one stably transformed expression cassette comprising the nucleotide sequence of interest. Alternatively, the host cell can be a stem cell or other early tissue precursor that gives rise to a specific subset of cells and can be used to produce transgenic tissues in an animal. See also Thomas et al., (1987) Cell 51: 503 for a description of homologous recombination vectors. The vector is introduced into an

Robertson, ed. (IRL, Oxford, pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the recombined DNA by germ line transmission of the Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) Current Opinion in Biotechnology 2: 823-829 and in PCT International Publication Nos. WO 90/11354; WO ; and WO

Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U. S. Pat. Nos. 4, 866; 4,870, 009; 4,873, ; 6,201, 165 and in Nagy et al Ed.

(2003) Cold Spring Harbor Press, Cold Spring Harbor, NY).

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature 385 : 810-813 and PCT cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter Go enucleated oocyte from an animal of the same species from which the quiescent cell is isolated.

The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to a pseudopregnant female foster animal. The offspring born of this female foster

Other examples of transgenic animals include non-human primates, sheep, dogs, pigs, guinea pigs, hamsters, cows, goats, rabbits, and rats. Methods for providing transgenic rabbits are described in Marian et (1999) J. Clin. Invest. 104: 1683-1692 and James et (2000) Circulation 101: 1715-1721.

By"PKCa activity"is intended any activity exhibited by the wild-type described herein. Such activities include, but are not limited to, kinase activity, receptor of activated C kinase (RACK) binding activity,

expression, translocation from the cytosolic fraction to the particulate fraction, and translocation to the sarcolemma. Modulation of activity includes but is not limited to modulation of a activity such as kinase activity, RACK binding, or modulation of expression levels or cellular distribution.

Methods of assaying kinase activity are known in the art and include, but are not limited to, immunoprecipitation with antibodies to phosphor-peptides; fluorescence polarization ; filter binding assays with radioisotopes, scintillation proximity assays, 96 well assays with conjugated antibodies; time resolved fluorescent assays, thin layer chromatography ; immunoprecipitation and immune complex assays; non-trichloroacetic acid phosphoamino acid determinations; and protein kinase assays. See Braz et (2002) 156: 905-919; Ping et (1999)

276: ; U. S. Patent Application No: 20030036106; U. S. Patent No: 5447860; Walker, John, ed. (2002) Protein Protocols on 2; and Ausubel et al., eds. (1995) Current Protocols in Molecular Biology, (Greene Publishing and Wiley-Interscience, New York).

Methods of analyzing association with RACKs are known in the art and include, but are not limited to, ELISA, protein interactive trapping, crystallography, NMR, ultracentrifugation, immunoprecipitation, co-immunoprecipitation, cross-linking, yeast two- hybrid assays, and affinity chromatography. See for example Mochly-Rosen (1995) Soc. Trans. 23 (3): 596-600; Walker, John, ed. (2002) Prot. ein Protocols on CD-ROMv. 2 ; and Ausubel et eds. (1995) Current Protocols in Molecular Biology, (Greene Publishing and Wiley-Interscience, New

The invention provides methods of monitoring translocation of a indicator polypeptide. By"indicator polypeptide"is intended any polypeptide suitable for monitoring location. Suitable indicator polypeptides include fusion polypeptides containing reporter genes described earlier, such as, but not limited to, fluorescent proteins (e. g. galactosidase, c-myc; affinity polypeptide tags (such as His tags), radiolabeled polypeptides, biotin labeled polypeptides, antigen labeled polypeptides, and dye labeled polypeptides. Suitable indicator polypeptides include antibodies specific to the polypeptide of interest.

In an embodiment, the invention provides a method of altering expression in an animal. In an embodiment, expression is modulated throughout the animal (e. g. the disruption mutant). In an embodirnent expression is modulated in a cardiac preferred manner. By"cardiac-preferred"is intended that expression of the heterologous is most abundant in cardiac tissue, while some expression may occur in other tissue types, particularly in tissues developmentally related to cardiac tissue.

Methods of determining expression levels are known in the art and include, but are not limited to, qualitative Western blot analysis, immunoprecipitation, radiological assays, polypeptide purification, analysis, Coomassie staining of acrylamide gels, RT-PCR, 2-D gel electrophoresis, microarray analysis, in situ hybridization, chemiluminescence, silver staining, enzymatic assays, ponceau S staining, multiplex assays, colorimetric analysis, immunoradiometric assays, positron emission Northern blotting, fluorometric assays and SAGE. See, for example, Ausubel et al, eds. (2002) Current Protocols in Molecular Biology, Wiley- Interscience, New York, New York; Coligan et al (2002) Current Protocols in Protein Science, Wiley-Interscience, New York, New and Sun et (2001) Gene 8: 1572-1579.

It is recognized that the nucleotide sequences may be used with their native promoters to increase or decrease expression resulting in a change in phenotype in the cardiac tissue of the transformed animal.

Transgenic animals that exhibit altered cardiac preferred expression of are useful to conduct assays that identify compounds that affect cardiac function such as, but not limited to, cardiac contractility. Assays to determine cardiac contractility are known in the art and include, but are not limited to, shortening

assays, peak shortening, time to peak, time to'/2 maximal relaxation, contracting and relaxing rate
assays, changes in cardiac chronotropy, changes in cardiac lusitropy, and gross heart contraction assays.
The altered cardiac-preferred expression of the expression may result in altered susceptibility to a
cardiomyopathy. In an aspect of the invention, the invention provides methods of acutely modulating
cardiac contractility. In another aspect of the invention, the invention provides methods of acutely
modulating a cardiomyopathy.

An acute modulation or alteration begins within 1 second; 10 seconds; 30 seconds; 1, 2, 3,4, 5,6, 7,8,
9,10, 11,12, 13,14, 15,20, 25,30, 35,40, 45,50, 60 minutes; 2,3, 4,5, 6,7, 8,9, 10, 11,12, 13,14, 15,16,
17,18, 19,20, 21,22, 23,24 hours; 2,3, 4,5, 6,7, 9,10, 12,13, 14, 15,16, 17,18, 19,20, 21,22, 23,24, 25,26,
27,28, 29,30, or 31 days after administration of the modulating agent. The duration of the modulation
ranges from short durations such as, but not limited to, nanosecond, second, and minute increments;
intermediate durations such as, but not limited to, hour, day, and week increments; to long durations
such as, but not limited to, month and year increments, up to and including the recipient's lifespan.

contractility"are defined as measures of cardiac function, which may include but are not limited to
cardiac output, ejection fraction, fractional shortening, cardiac work, cardiac index, chronotropy,
lusitropy, velocity of circumferential fiber shortening, velocity of circumferential fiber shortening
corrected for heart rate, stroke volume, rates of cardiac contraction or relaxation, the first derivatives of
interventricular pressure (maximum dP/dt and minimum dP/dt), ventricular volumes, clinical
evaluations of cardiac function (for example, stress echocardiography and treadmill walking) and
variations or normalizations of these parameters. These parameters may be measured in humans or
animals alike to assess myocardial function and assist in diagnosis and prognosis of heart disease.

A"cardiomyopathy"is any disorder or condition involving cardiac muscle tissue or cardiac dysfunction.
Disorders involving cardiac muscle tissue include, but are not limited to, myocardial disease, including
but not limited to dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy,
myocardial stunning, and myocarditis; heart failure ; acute heart failure; rheumatic fever; rhabdomyoma;
sarcoma; congenital heart disease, including but not limited to, left-to-right shunts--late cyanosis, such
as atrial septal defect, ventricular septal defect, patent ductus arteriosus, and atrioventricular septal
defect, right-to-left shunts--early cyanosis, such as tetralogy of fallot, transposition of great arteries,
truncus arteriosus, tricuspid atresia, and total anomalous pulmonary venous connection, obstructive
congenital anomalies, such as coarctation of aorta, pulmonary stenosis and atresia, and aortic stenosis
and atresia; disorders involving cardiac transplantation; arterial hypertension; peripartum
cardiomyopathy ; alcoholic cardiomyopathy; tachycardias ; supraventricular tachycardia ; bradycardia;
atrial flutter; hydrops fetalis; arrhythmias; extrasystolic arrhythmia; fetal cardiac arrhythmia;
endocarditis; atrial fibrillation; idiopathic dilated cardiomyopathy; ; long QT syndrome; Brugada
syndrome; ischemia; hypoxia; ventricular fibrillation ; ventricular tachycardia ; restenosis; congestive
heart failure; syncope; arrythmias; pericardial disease; myocardial infarction; unstable angina; stable
angina; and angina pectoris, viral myocarditis, and non-proliferating cell disorders involving cardiac
muscle tissue.

By"altered susceptibility"is intended that a transgenic animal of the invention differs from a non-
transgenic animal in the extent to which the transgenic animal of the invention exhibits a
cardiomyopathic phenotype. The cardiomyopathic phenotype may present during any stage of
development including, but not limited to, embryonically, post-natally, in the adult, and as the animal
nears end of lifespan. In an embodiment, the cardiomyopathic phenotype may be induced by external
stimuli such as, but not limited to, diet, exercise, chemical treatment, or surgical procedure.

phenotypes include, but are not limited to, hypertrophy ; morphology, such as interventricular septal
hypertrophy; left ventricular-end systolic maximum or end- diastolic ; papillary muscle dimension; left-

ventricular outflow tract obstruction; midventricular hypertrophy; apical hypertrophy; asymmetrical hypertrophy; concentric enlarged ventricular mass; eccentric enlarged ventricular mass; sarcomere structure; myofibril function; receptor expression; heart rate; ventricular systolic pressure; ventricular diastolic pressure; aortic systolic pressure; aortic diastolic pressure ; contractility ; interstitial fibrosis; cardiomyocyte disarray; $Ca2+$ sensitivity; $Ca2+$ release ; $Ca2+$ uptake ; catecholine sensitivity; a-adrenergic sensitivity; beta-adrenergic sensitivity; dobutamine sensitivity; thyroxine sensitivity; angiotensin- converting enzyme inhibitor sensitivity; amiodarone sensitivity; lidocaine sensitivity; glycoprotein receptor antagonist sensitivity; anabolic steroid sensitivity; carnitine transport irregularities; left ventricular dilation, reduced left ventricular ejection fraction; left atrial dilatation ; diuretic sensitivity; volemia; ischemia ; leukocyte flow properties; the polymorphonuclear leukocyte membrane fluidity; PMN cytosolic $Ca2+$ content ; high interventricular septal defects, rosette inhibition effect; contractile force transmission; myocardial fiber disarray; increased chamber stiffness ; impaired relaxation; small-vessel disease; dyspnea; angina; ; tachycardia; syncope; lethargy; respiratory distress; ruffled fur; hunched posture; peripheral edema; ascites; hepatomegaly; edematous lung; cardiomegaly; organized thrombi formation; heart weight ratio; rate of pressure development, rate of pressure fail, cell twitch measurement and the like. See, for example, Braunwald et (2002) Circulation 106: 1312-1316; Wigle et (1995) Circulation 92: 1680-1692; and Pi & Walker (2000) Ain. J.

279: H26-H34; hereby incorporated by reference in their entirety.

Methods for measuring cardiomyopathic phenotypes are known in the art and include, but are not limited to, trans-thoracic echocardiography, transesophageal echocardiography, exercise tests, urine/catecholamine analysis, EIAs, light microscopy, heart catheterization, dynamic Langendorff hanging heart preparation, working heart preparation, MRI, multiplex RT-PCR, positron emission tomography, angiography, magnetic resonance spin echo, short-axis MRI scanning, Doppler velocity recordings, Doppler color flow imaging, stress thallium studies, cardiac ultrasound, chest X-ray, oxygen consumption test, electrophysiological studies, auscultation, scanning EM, gravimetric analysis, hematoxylin and eosin staining, skinned fiber analysis, transmission electron microscopy, analysis, trichrome staining, Masson's trichrome staining, Von Kossa staining, 2-D echocardiography, cardiotocography, baseline M-mode echocardiography, and myocardial lactate production assays. See, for example, Braz et al. (2002) Cell. Biol. 156: 905-919; Braunwald (2002) Circulation 106: 1312-1316; Sohal et al. (2001) Circulation Res. : 20-25; et al. (2000) Circulation 102: 1346-1350; Sanbe et (2001) J. Biol. 276: 32682-32686; Sanbe et al. (1999) Biol. Chez.

274: 21085-21094 ; Wigle et (1995) Circulation 92: 1680-1692; Pi & Walker (2000) Am.

Physio. 279: H26-H34; and Wang et al. (2001) Am. J.

Physio. 269: H90-H98 hereby incorporated by reference in their entirety.

The used herein to mean that, at a minimum, administration of a compound of the present invention mitigates a disease or a disorder in a host, preferably in a mammalian subject, more preferably in humans. Thus, the term"treatment"includes: preventing an infectious disorder from occurring in a host, particularly when the host is predisposed to acquiring the disease, but has not yet been diagnosed with the disease; inhibiting the infectious disorder; and/or alleviating or reversing the infectious disorder. Insofar as the methods of the present invention are directed to preventing disorders, it is understood that the does not require that the disease state be completely thwarted. (See Webster's Ninth Collegiate to identify a population that is susceptible to disorders, such that administration of the compounds of the present invention may occur prior to onset of a disease. The term does not imply that the disease state be completely avoided.

Identification inhibitors for the treatment of impaired cardiac contraction and relaxation can be identified by known methodologies. inhibitors could be identified by assessing the enzymatic activity of This may be accomplished by using a number commercially available kits. Some of these kits use"labeled"substrates, including, but not limited to luminescent, fluorescent, radioactive or other measurable and quantifiable endpoints.

Alternatively, as set forth in this invention, the protein itself could be attached to a traceable marker, including, but not limited to luminescent, fluorescent or radioactive ion or molecule in order to determine the distribution and activity of in isolation or in a cell or tissue. As has many known substrates in the cell, activity could be assessed by measuring the phosphorylation or dephosphorylation of substrates. substrates phosphorylation/dephosphorylation status may be measured using labeled or unlabeled phosphorylation site-specific antibodies, luminescent, fluorescent, radioactive biological labels or other means to assess activity against its substrates. Alternatively, the redistribution of the substrate (s) may also serve as a means of measuring its response to alterations in activity.

In the case where the substrate is a kinase, phosphatase or other enzyme, the activity of the substrate may be measured by established techniques.

Identification of inhibitors that would be beneficial in humans with cardiac dysfunction may be accomplished using isolated cells or isolated tissues in which it has been determined that present. For instance, PKCa inhibitors may be tested in isolated cells, preferably cardiomyocytes, from mammals or other organisms and determine the effect of inhibitors by measuring the percent shortening of the cell (% FS): the rates of shortening or re- lengthening by standard techniques (Chaudhri B et al. (2002) Am JPhysiol Heart Circ 283: H2450-H2457). Alternatively, muscle (s), preferably of cardiac origin, may be isolated and measurements of contractile function assess in the presence and absence of inhibitors, by standard techniques (Slack JP et al. (1997) 272: 18862-18868). inhibitors may be identified as outlined in the present invention by measuring acute hemodynamics, including heart rate, blood pressure, rates of contraction and relaxation and-dP/dt), left ventricular pressure and derivations of these parameters. inhibitors may be identified by these methods in suitable, normal animals including, but not limited to, various genetic strains of mice, rats, guinea pigs, hamsters, humans, rabbits, dogs, pigs, goats, cows, monkeys, chimpanzees, sheep, hamsters and zebrafish. inhibitors could be identified by these methods in suitable, animal models of heart failure or cardiac dysfunction including, but not limited to, various genetic strains of transgenic or knockout mice, such as the KO mice, serine/threonine phosphatase overexpressing mice and overexpressing transgenic mice. In addition, inhibitors may be identified in spontaneous or natural models of heart failure and cardiac dysfunction due to a genetic or multiple genetic defects, including but not limited to the spontaneous hypertensive heart failure rat or the Dahl salt sensitive rat. In addition, inhibitors may be identified in surgically induced models of cardiac dysfunction including, but not limited to, myocardial infarction models, coronary model, aortic constriction model, arteriovenous fistula model or other pressure or volume overload models in rats, guinea pigs, rabbits, dogs, pigs, goats, cows, monkeys, chimpanzees, sheep, hamsters and zebrafish.

In an embodiment, a transgenic animal, tissue, or cell of the invention may be used to identify modulating compounds. modulating compound"is a compound that modulates a activity. modulating compounds include, but are not limited to, diacylglycerols, phosphatidylserine, Ca^{++}; PMA, CGP54345, bisindolylmaleimide, CGP 54345, (also and Ro-32-0432 (also Methods for assaying activity are described elsewhere herein. Any method of assaying a activity known in the art may be used to monitor the effects of the compound of interest on a transgenic animal of the invention.

inhibitors include, but are not limited to, kinase inhibitors, protein kinase C inhibitors, and specific inhibitors. By"kinase inhibitor"is intended a compound that inhibits multiple kinases including By"protein kinase C inhibitor"is intended a compound preferentially inhibits activities of a protein

kinase C as compared to its effect on other kinases. specific inhibitor"is intended a compound that reduces a activity more than it reduces an activity of another kinase, including other protein kinase C isozymes. Known inhibitors include, but are not limited to, nucleic acid molecules having antisense nucleotide sequences and antisense molecules commercially available from lsis and dominant negative mutations of such as the lysine 368 arginine mutation (Braz et al.

(2002) J. Cell. Biol. 156: 905-919).

Antisense constructions complementary to at least a portion of the messenger RNA for a nucleotide sequence can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding In this manner, antisense constructions having at least about 70%, preferably at least about 80%, more preferably at least about 85% sequence identity to the corresponding sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used. Thus, antisense DNA sequences may be operably linked to a cardiac tissue-preferred promoter to reduce or inhibit expression of a native protein in cardiac tissue.

In addition to antisense technologies, gene expression can be repressed by double stranded RNA including short-hairpin RNA (shRNA), RNA interference (RNAi), short terminal RNA (stRNA), mikroRNA (miRNA) or short interfering RNA (siRNA) (Schutze N. (2004) Mol Cell These RNA interfering approaches can use RNA of varying sizes, but are generally limited to 15-28 nucleotides and act by an as yet unclear mechanism. This technique has been successfully employed in vitro and in vivo as a means to inhibit gene functions (McCaffrey AP et al. (2002) Nature 418,38-39).

Criteria evaluated for augmented contractility and heart failure progression include, but are not limited number, p-receptor coupling, adenylyl cyclase activity, cAMP levels at rest, cAMP levels after forskolin administration, PKA activity, PKA protein levels, L-type calcium channel current density, SERCA2a protein levels, and phospholamban levels, or phospholamban phosphorylation of proteins.

Compounds that can be screened in accordance with the assays of the invention include but are not limited to, libraries of known compounds, including natural products, such as plant or animal extracts, synthetic chemicals, biologically active materials including proteins, peptides such as soluble peptides, including but not limited to members of random peptide libraries and combinatorial chemistry derived molecular library made of D-or L-configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries), antibodies (including, but not limited to, polyclonal, monoclonal, chimeric, human, anti-idiotypic or single chain antibodies, and Fab, F and Fab expression library fragments, and epitope-binding fragments thereof), organic and inorganic molecules.

In addition to the more traditional sources of test compounds, computer modeling and searching technologies permit the rational selection of test compounds by utilizing structural information from the ligand binding sites of proteins of the present invention. Such rational selection of test compounds can decrease the number of test compounds that must be screened in order to identify a therapeutic compound. Knowledge of the sequences of proteins of the present invention allows for the generation of models of their binding sites that can be used to screen for potential ligands. This process can be accomplished in several manners known in the art. A preferred approach involves generating a sequence alignment of the protein sequence to a template (derived from the crystal structures or NMR-based model of a similar protein (s), conversion of the amino acid structures and refining the model by molecular mechanics and visual examination. If a strong sequence alignment cannot be obtained then a

model may also be generated by building models of the hydrophobic helices. Mutational data that point towards residue-residue contacts may also be used to position the helices relative to each other so that these contacts are achieved. During this process, docking of the known ligands into the binding site cavity within the helices may also be used to help position the helices by developing interactions that would stabilize the binding of the ligand. The model may be completed by refinement using molecular mechanics and loop building using standard homology modeling techniques. General information regarding modeling can be found in Schoneberg, T. et. Molecular and Cellular Endocrinology, 15 1 : 181-193 (1999), Flower, D., Acta, 1422: 207-234 (1999), and Sexton, P. M., Current and Development, 2 (5): 440-448 (1999).

Once the model is completed, it can be used in conjunction with one of several existing computer programs to narrow the number of compounds to be screened by the screening methods of the present invention, like the DOCK program (UCSF Molecular Design Institute, 533 Parnassus Ave, U-64, Box 0446, San Francisco, California 94143-0446). In several of its variants it can screen databases of commercial proprietary compounds for steric fit and rough electrostatic complementarity to the binding site. Another program that can be used is FLEXX

As used herein the language"pharmaceutically acceptable carrier"is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions. A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

carboxypeptidase protein or anti-carboxypeptidase antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze- drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For oral administration, the agent can be contained in enteric forms to survive the stomach or further coated or mixed to be released in a particular region of the GI tract by known methods. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel (RTM), or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives.

Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U. S. Pat. No. 4,522, 811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in

association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Anti-cardiomyopathic compounds identified by the methods of this invention may be used in the treatment of humans.

METHODS Example Generation of Transgenic Mice The gene was targeted for deletion by standard homologous recombination in embryonic stem cells, followed by production of chimeric mice, which were bred and passed the targeted allele into the germline. The exon encoding the ATP binding cassette in was deleted resulting in a null allele with regards to protein expression. For generation overexpressing transgenic mice, a encoding was into the murine a- myosin heavy chain promoter-containing expression vector and injected into newly fertilized oocytes. The MLP, and pressure-overload surgical model (TAC) were all described elsewhere et al. (1997) Cell 88 : 393-403; Carr et (2002) 22: 4124-4135; and Liang et al. (2003) EMBO. J. 22: 5079-5089). Males were exclusively used in all studies for consistency. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Example 2. Analysis Mice from all genotypes or treatment groups were anesthetized with isoflurane, and echocardiography was performed using a Hewlett Packard 5500 instrument with a 15-MHZ microprobe. measurements were taken on M-mode in triplicate from four separate mice per group. The isolated ejecting mouse heart preparation used in the present study has been described in detail previously (Gulick et (1997) Circ. Res. 80: 655-664), as was the close-chested working heart model employed here (Lorenz et al. (1997) Physio.

272:

Example 3. Marker Gene Analyses Hearts were collected at the indicated times, fixed in 10% formalin containing PBS, and embedded in paraffin. Serial 5-um heart sections from each group were analyzed. Samples were stained with hematoxylin and eosin or Masson's trichrome. Cardiac gene expression of hypertrophic molecular markers was assessed by RNA dot-blot analysis as described previously (Jones et al. (1996) : 1906-1917).

Example in Single Adult Rat Cardiac Myocytes after Adenoviral Infection Ventricular myocytes were isolated from Sprague-Dawley rat hearts (Westfall et (1997 Methods Cell Biology 52: 307-322), and plated on laminin-coated coverslips in DMEM with 5% serum for 2 hr. Media was then replaced with serum-free DMEM containing a recombinant viral vector. Serum-free DMEM was added after 1 hr, and media was changed every 2 days. About 70-85% of isolated cells are rod shaped, with rod-shaped myocytes per heart. Myocytes used for shortening assays were electrically stimulated in media 199 supplemented with Penicillin/Streptomycin, 10 mM Hepes, 0.2 mg/ml albumin, and 10 mM glutathione (Westfall and Borton, (2003) J. Chem. 278: 33694-33700). Myocytes were transferred to a stimulation chamber with platinum electrodes 1 day after plating, and stimulated at 0.2 Hz with a 2.5 ms pulse at a voltage producing twitches in <25% of myocytes. Media in the stimulation chamber was replaced every 12 hrs. For contractile function studies, coverslips were mounted in a thermo- controlled chamber containing for sarcomere shortening measurements. Sarcomere length was measured via a variable field rate CCD video camera (Ionoptix ; Milton, MA), and recorded with sarcomere length detection software. Myocytes were stimulated at 0.2 Hz, and sarcomere shortening was recorded for 60 sec. Measurements of peak shortening, time to peak, time to half maximal relaxation and contraction plus relaxation rates were obtained from the signal average for 10 contractions. Results from these studies were compared by a one-way analysis of variance and a post-hoc Newman-Keuls test.

Example 5. Electrophysiological Recordings Cardiac myocytes were dissociated from the ventricles of 3-month-old wildtype or non- transgenic (Ntg) and mice and electrophysiological recording were performed as described before (Petrashevskaya et al. (2002) Cardiovasc. Res. 54: 117-132 and et al.

(1997) Ana. J. Physio. 272: H606-H612). Briefly, the heart was subject to retrograde coronary perfusion with Ca2+-free Tyrode's solution for 10 minutes, and with Tyrode's solution (250 uM containing collagenase type II (Worthington; 1.0 mg/ml) supplemented with 5 mM taurine and 10 mM BDM (2,3-butane, dione-monoxamine) for 8-12 minutes at bubbled with 95% and 5% At the end of the perfusion the heart was removed and the ventricular tissues were mechanically minced in low high medium. The minced ventricular tissue was then gently filtered, and stored at until electrophysiological study. Only tolerant cells with clear cross striations and without spontaneous contraction or significant granulation were selected for experiments.

Experiments were performed on the dissociated cardiac myocytes at 20 to All current recordings were obtained in the whole cell, voltage-clamp configuration of the patch clamp technique by using 1.60 OD borosilicate glass electrodes (Garner Glass Company). Cell capacitance was calculated by integrating the area under an uncompensated capacity transient elicited by a 25 mV hyperpolarizing test pulse (25 ms) from a holding potential of 0 mV.

Resistance was within the range of 2 to 11 Most of the data presented in these studies were obtained with electrodes having a resistance of 0.5-3 After formation of a high resistance seal between the recording electrode and the myocyte membrane, electrode capacitance was fully compensated electronically before breaking the membrane patch. currents were elicited by depolarizing voltage steps (380 ms) from-50 to +40 mV in 10 mV increments from a holding potential-60 mV. The recorded currents were filtered at 2 kHz through a four-pole low- bass Bessel filter and digitized at 5 kHz. The experiments were controlled using pClamp 5.6 software (Axon Instruments) and analyzed using 6.0. 3. currents were recorded using an external solution containing (in : tetraethyl-ammonium chloride (TEA-Cl) 4-aminopyridine (4-AP) 5, glucose 10, HEPES 10, (pH 7.3). The pipette solution contained (mM): cesium aspartate 100, 20, 1, 2, Na2-GTP 0.5, EGTA 5, HEPES 5, (pH 7.3 with These solutions isolated from other membrane currents such as and currents and also Ca2+ flux through the exchanger.

Example 6. Calcium Transient Measurements Isolation of mouse left ventricular myocytes for assessment of calcium transient measurements was carried out as described previously (Chu et al. (1996) Res. 79: 1064- 1076). transients were measured from cardiomyocytes at room temperature. Briefly, mouse in a Langendorff perfusion apparatus, and perfused with Tyrode solution at for 3 min. The normal Tyrode solution contained 140 mM NaCl, 4 mM KCl, 1 mM glucose, and HEPES, pH 7.4. Perfusion was then switched to the same solution containing 75 units/ml type 1 collagenase (Worthington), and perfusion continued until the heart became flaccid The left ventricular tissue was excised, minced, pipette-dissociated, and filtered through a screen. The cell suspension was then sequentially washed in 25,100, 200 AM and 1 mM To obtain intracellular Ca2+ signals, cells were incubated with the acetoxymethyl ester form of fura-2 (Fura-2/AM; 2 for 30 min at room temperature and resuspended in 1.8 mM Ca2+-Tyrode solution. The myocyte suspension was placed in a Plexiglas chamber, which was positioned on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200), and perfused with 8 mM solution at room temperature

Myocyte contraction was field-stimulated by a Grass S5 stimulator (0.5 Hz, square waves), and the cells were alternately excited at 340 and 5 by Delta Scan dual-beam spectrophotofluorometer (Photon Technology International). Ca2+ transients were recorded as the 340/380 nm ratio of the resulting 510 nm emissions. Baseline and amplitude, estimated by the ratio, and the times for 80% decay of the Ca2+ signal and tau were acquired. All data were analyzed using software from FeliX and

Example 7. Phosphorylation PKC kinase reaction mixtures included 10 uM inhibitor-1, 20 mM MOPS, pH 7.2, 25 mM phosphate, 1 mM mM sodium mM DTT, mM 0.1 mg/ml phosphatidylserine, 0.01 diacylglycerol, uM ATP, and 0.6

PKC was isolated from rabbit heart muscle (Woodgett and Hunter, 1987) J.

262 and recombinant wildtype and Ser-67-Ala inhibitor-1 was isolated from (Bibb 276: 14490-14497). Reactions were conducted at 30°C and aliquots were removed at specific time points and stopped by addition of protein sample buffer.

Stoichiometries were determined by and direct quantification of radioactivity.

Example 8. Primary Cell Culture Primary cultures of neonatal rat cardiomyocytes were obtained by enzymatic dissociation of 1-2 day-old Sprague-Dawley rat neonates as described previously (De Windt et al. (2000) 275: were cultured under serum-free conditions in M199 media supplemented with penicillin/streptomycin (100 U/ml) and L-glutamine

Example 9. Replication The characterization of adenovirus-encoding wildtype or dominant negative mutants in cardiomyocytes was described previously (Braz et al. (2002) J. Cell Biol. 156: 905-919).

The dominant negative consisted of a lysine to arginine mutation in the ATP binding domain at amino acid position Each recombinant adenovirus was plaque purified, expanded, and titered in HEK293 cells. Typical experiments involved infection of 6 neonatal rat cardiomyocytes at a moi of 100 plaque forming units for 2 h at in a humidified, 6% incubator. Subsequently, the cells were cultured in serum-free media for an additional 24 h before analysis. Under these conditions 95% of the cells showed expression of the recombinant protein.

Example 10. PKC Translocation Assay and Immunoblot Analysis Soluble and particulate fractions were prepared as described previously (Braz et al.

(2002) J. Cell Biol. 156: 905-919). Protein samples were subjected to SDS-PAGE (10% gels), transferred to Hybond-P membrane (Amersham Pharmacia Biotech), blocked in 7% milk, and incubated with primary antibodies against SERCA2, calsequestrin, PLB, phospho- serine-16 PLB, inhibitor-1, and Phospho-specific were described previously (Bibb et al. (2001) Biol. 276: Primary antibodies were incubated overnight in milk at Secondary antibodies IgG (alkaline phosphatase-conjugated anti-

Chemifluorescent detection was directly performed with the Vistra ECF reagent (RPN 5785 ; Amersham Pharmacia Biotech) and scanned with a PhosphorImager or chemiluminescence was performed with (Amersham Pharmacia Biotech) and exposed on film.

Example 11. Immunoprecipitation and Protein Phosphatase Activity Assays Protein extracts were generated from cardiomyocytes infected with adenovirus encoding B-galactosidase, and Extracts were immunoprecipitated with PPIca conjugated to agarose beads, followed by western blotting against I-1. Preparation of phosphorylated protein substrate and radioactive assay of protein phosphatases were prepared as instructed by the Protein Serine/Threonine Phosphatase (PSP) Assay System (New England BioLabs, Inc.).

Example 12. Caffeine Induced Calcium Transients Caffeine induced calcium transients were measured in a total of 37 myocytes from 4 null mice and 19 control myocytes from 3 wild type mice. After collagenase digestion, myocytes were loaded with AM (25 ml) for 12 min at room temperature.

calcium transients (measured by Indo-1 fluorescence ratio) were recorded at resting state (no electrical stimulation) before and during 20 mM caffeine addition.

Example 13. Cardiac Functionality Assessment Hearts were isolated from four wild-type and four PKCa-/- null) transgenic mice.

The isolated hearts were infused with PMA at 9 different concentrations ranging from through 8 X Acute PMA infusion of each concentration occurred for a 7 minute period.

The hearts were measured for maximal and minimal dP/dt in systole and diastole respectively.

Results from one such experiment are presented in Fig. 36.

Example 14. PKC Isozyme Abundance Assessment A standard curve was used to assess the relative abundance of the PKC isozymes in healthy human hearts. Recombinant human protein PKCa, and generated in bacteria were purchased from a commercial vendor. Three aliquots of known concentrations were prepared.

Adult human ventricular tissue was explanted from six undiseased individuals. Whole cell protein lysates were prepared. The three standard PKC aliquots and the heart proteins were subjected to polyacrylamide gel electrophoresis on the same gel. The proteins were transferred to a membrane. The membrane was blocked and incubated with antibodies specific to the and PKCs isozymes. Data from such an experiment are presented in Figure 37.

Example 15. Cardiac The relatively selective inhibitory compound yl) maleimide, HCI Salt] 8,9-tetrahydropyrido [1, 10-yl}-4- (1-methylindol-3-yl)-1H-pyrrole-2, 5-dione, Salt] was used as a means of directly examining the effects of acute inhibition on cardiac function and contractility using an ex vivo working heart preparation. The working heart preparation separates the inherent pump function of the heart from potential alterations in total vascular resistance as might occur if the drug were infused in vivo.

Working adult wildtype mouse hearts were infused with vehicle control (10% DMSO) or Ro-32-0432 in 10% DMSO at concentrations ranging between through Four animals were analyzed in the Ro-32-0432 group and compared with three animals in the vehicle control group. Values throughout the concentration time course (7 minutes per 10 different incremental concentrations) were summated for statistical purposes, representing an average dosage of approximately M. The vehicle control and experimental groups showed heart rates of 363 +/-15 and 295 +/-26 beats per minute, respectively, before any treatments were begun. The average heart rate of the vehicle and drug treated groups was 351 +/= 3 and 292 +/-6 beats per minute, respectively. Despite the lower heart rate, the Ro-32-0432 infused group showed an increase in acute contractile function measured as maximum dP/dt, and an increase in left ventricular pressure developed. The approximate 20% change in acute contractile performance in the Ro-32-0432 treated group is similar to the increase in cardiac function observed in null mice. The data from one such experiment are presented in Figure 38.

Example 16. Translocation of a Indicator Polypeptide A indicator was prepared by operably linking a nucleotide sequence encoding to a nucleotide sequence encoding green fluorescence protein (GFP). An expression cassette comprising the nucleotide sequence was prepared. Adenovirus comprising the expression cassette was prepared.

Neonatal rat cardiomyocytes were cultured in plastic dishes and incubated until the appropriate density

was reached. The cardiomyocytes were infected with an adenovirus encoding The cultures were incubated for 24 hours. After 24 hours the cells were incubated with either DMSO alone (the vehicle treatment) or DMSO and PMA for 60 minutes. The cells were fixed and examined by confocal microscopy. The PMA stimulated cells exhibit a highly localized and punctate staining pattern whereas the vehicle only stimulated cells exhibit a relatively diffuse localization.

Example 17. In Vivo Evaluation of Inhibitors in the Anesthetized Rat.

Selected inhibitors are evaluated in both naive rats and rats with myocardial infarction (MI) for effects on cardiac contractility and hemodynamics.

Male, Sprague-Dawley or Lewis rats weighing between 225-500 gm are anesthetized with isoflurane and an MI is induced as follows. A thoracotomy at the fourth or fifth intercostal space is done, the heart is exposed and the pericardium is opened. A 5-0 suture is placed around the left descending coronary artery 2-4 mm from its origin and permanently tied. The ribs, muscle and skin are separately closed and the animal is allowed to recover. Twenty to twenty-three weeks after surgery the animals are used to evaluate the effects of inhibitors on cardiac contractility and hemodynamics.

The effects of inhibitors on cardiac contractility and hemodynamics are evaluated in naive and MI rats as follows. The animals are anesthetized with isoflurane. A femoral artery is isolated and cannulated for the measurement of systemic blood pressure. A jugular vein is isolated and for the intravenous infusion of inhibitor. The right carotid artery is isolated and a Millar conductance catheter is inserted to the left ventricle (LV) of the heart. The LV systolic pressure, end-diastolic pressure, +maximum dP/dt,- minimum and heart rate are derived from the LV pressure waveform. Mean arterial blood pressure is derived from the systemic blood pressure Data are recorded continuously and derived using computerized data acquisition software (Notocord or Powerlab).

After a period of stabilization, inhibitors are infused at the following infusion doses in naive rats: 0.1, 0.3, 1.0, 3.0, 10,30, 100,300 and 1000 nmol/kg/min. The infusion of each dose is allowed to run for at least five minutes. In MI rats, the infusion doses 10,30, 300, and 1000 nmol/kg/min for at least five minutes. Equivalent infusion volumes are administered to separate, vehicle-control naive and MI animals. At the end of the test infusion, 5.0 is infused.

Example 18. Method of This assay can be used for a variety of cardiomyopathic phenotypes. A nucleotide sequence of interest is cloned into an expression vector containing a cardiac tissue-preferred promoter. The expression cassette comprising the promoter, operably linked to the nucleotide sequence of interest is digested with a restriction enzyme. The restriction reaction products are electrophoresed on an agarose gel, and the expression cassette is purified from the agarose. The expression cassette is prepared for microinjection according to any method known to one skilled in the art. The expression cassette is used to provide a transgenic mouse. The presence of the transgene is confirmed using Southern blot analysis.

Two cohorts of age-matched transgenic mice are established. The diet of one cohort is supplemented with a compound of interest. The diet of the second cohort is supplemented with a placebo. The two mice cohorts are incubated for an appropriate time and the experiment is terminated. The mice are monitored for a cardiomyopathic phenotype such as hypertrophy using the left mass ratios described elsewhere herein. A cardiomyopathic phenotype presented by the mice of the each cohort is compared. Alternatively, the compound may be administered directly to the animals using established methodologies and technologies, including but not limited to, intra-arterial or intravenous injection of a compound by syringe or osmotic mini-pumps or other means, oral gavage, intraperitoneal injection or

subcutaneous injection.

EXPERIMENTAL RESULTS AND DISCUSSION In the following figures, data are presented with the standard error of the mean, unless otherwise indicated.

Figure The locus (also called Prkca) was targeted by homologous recombination in embryonic stem cells so that the exon encoding the catalytic ATP binding cassette was deleted by replacement with the neomycin resistance marker (shown in panel A). Genomic targeting was detected by Southern blotting with EcoRV digested DNA and a 5'probe external to the region of vector homology (shown in panel B), demonstrating correct targeting and deletion of the selected exon. Correctly targeted embryonic stem cells were used to generate germline-containing targeted mice using common techniques routinely employed in the previous art. were intercrossed, generating at the predicted Mendelian frequencies. Panel C shows western blotting for protein levels from heart protein extracts derived from wildtype, demonstrating that protein is completely eliminated in PKCa-/-mice and reduced by approximately 50% in compared with non- targeted wildtype mice.

Figure 2. To evaluate the potential that other PKC isozymes might compensate for the loss of in the heart, western blotting was performed from hearts from 2 month-old mice subjected to pressure-overload by transverse aortic constriction (TAC) for 2 weeks, or sham control animals. Wildtype control animals were also subjected to TAC or sham operations.

Protein extracts from these hearts were separated into soluble (S) or particulate (P) fractions and western blotted for select PKC isozymes. The data demonstrate that completely lack protein, while and levels or translocation efficiencies were unaffected.

These results indicate that alternate PKC isozymes are unlikely to overtly compensate for the loss of in the heart.

Figure 3. Close-chested invasive hemodynamic assessment of 6 6 non- targeted wildtype mice demonstrated a 15-20% increase in maximum dP/dt at baseline, with a corresponding parallel increase in performance following receptor stimulation with dobutamine. These results indicate that have hypercontractile hearts in vivo.

- Figure 4. To assess the intrinsic function of the heart apart from potential hemodynamic compensatory responses, an ex vivo anterograde working heart preparation was performed at 2 and 10 months of age from wildtype mice (4 hearts in each group). Each heart was paced at approximately 400 beats per minute to ensure equal assessment of functional capacity.

PKCa-l-hearts showed a 15% and 32% increase in maximum dP/dt at 2 and 10 months, respectively, compared to age-matched, wildtype controls (panel A). A corresponding increase in left ventricular pressure development was also observed in (panel B).

These results further indicate that have hypercontractile hearts and the defects are not compensated by other mechanisms found in the whole animal.

Figure 5. Close-chested invasive hemodynamic assessment of 6 6 non- targeted wildtype mice demonstrated no change in heart rate (panel A) or mean arterial blood pressure (panel B). These results indicate that the increase in contractility observed in PKCa-/- hearts is not due to a secondary alteration in blood pressure or heart rate.

Figure 6. To assess the gain-of-function phenotype associated with protein ablation in the heart, transgenic mice were generated that overexpress the wildtype under the control of the cardiac-specific a-myosin heavy chain promoter (panel A). Quantitative western blotting from heart protein extracts of wildtype mice or the PKCa-overexpressing transgenic mice demonstrated 5-fold overexpression of protein in transgenic hearts, without any compensatory changes in (panel B, upper). Western blotting of cardiac protein extracts derived from wildtype, transgenic, or PKCa-/-mice showed increased autophosphorylation of suggesting greater activity due to the (panel B, lower).

extract was used as a migration control in the western blotting procedure. These results indicate that transgenic mice have significantly greater activity in the heart.

Figure 7. transgenic mice manifest signs of cardiomyopathy. By 4 months of age, PKCa transgenic mice show reduced fractional shortening as measured by echocardiography compared to age and strain-matched wildtype controls, suggesting that increased activity diminishes cardiac contractile performance in vivo (panel A). This conclusion is also supported by assessment of maximal dP/dt as assessed by an ex vivo working heart preparation (panel B), which also shows reduced cardiac functional performance in transgenic mice compared with wildtype controls. Four animals were used in each group (A & B) in the above experiments.

Figure 8. transgenic mice did not manifest signs of cardiac hypertrophy until 6 and 8 months of age, a time slightly after reduced contractile performance was noted in Figure 7. The gradual manifestation of cardiac hypertrophy by 6 and 8 months of age is a consequence of reduced contractile performance, that together indicates that enhanced activity in the heart produces cardiomyopathy. Four animals were used in each group.

Figure 9. While gene-targeted and transgenic mice demonstrated an antithetic cardiac contractility phenotype, the potential for secondary effects associated with a chronic alteration in activity cannot be disregarded. To address this concern an acute model of PKCa activation or inhibition was instituted in wildtype adult rat cardiac myocytes, followed by examination of single cell contractile responsiveness. gene transfer of wildtype or dominant negative reduced and enhanced myocyte contractility, respectively, as measured by peak shortening (P<0.05). Maximal shortening velocity was also similarly affected, with values of 4.04 23 in control adult myocytes compared to 3. 25 and 5. 480. 36 in wildtype and dominant negative adenoviral-infected myocytes, respectively (P<0.05). These data indicate that acute alterations in activity impact myocyte contractility, consistent with the genetic mouse models presented in Figures 1-8.

Figure 10. PKCa-l-hearts showed a hyperphosphorylation of phospholamban (PLB) resulting in retarded migration by western blotting and an increase in direct phosphorylation at serine-16, without a change in SERCA2 or calsequestrin protein levels (panels A, B). The pentameric form of PLB is shown to more represent differences in migration. Interestingly, the observed profile of PLB was also associated with reduced PLB protein levels so that the PLB/SERCA2 protein ratio was reduced by 50-70%, which is predicted to render SERCA2 more active (panel Direct measurement of PLB phosphorylation at serine 16, the site know to alter the contractile effectiveness of PLB, was increased in the hearts of PKCa-/-mice compared with wildtype (Wt) control hearts (panels C, D). These results indicate a potential mechanism whereby loss protein enhances cardiac contractile performance through diminished PLB effectiveness in inhibiting SERCA2a activity.

Figure 11. The overall regulatory paradigm between and PLB was also observed in acutely infected adult rat cardiomyocytes. Specifically, dominant negative expression by adenoviral-mediated gene transfer was associated with increased phosphorylation of PLB at serine-16. Collectively, these results indicate that alterations in signaling impacts PLB phosphorylation status and protein levels, suggesting a

mechanism whereby ablation or overexpression might affect contractility.

Figure 12. No change in PLB or SERCA2 levels were detected between wildtype and PKCa-l-hearts by RNA dot blotting (panel A) or semiquantitative RT-PCR (panel B), suggesting that the observed down-regulation of PLB protein presented in Figure results from a post-transcriptional mechanism. This decrease in PLB protein levels is hypothesized to result from decreased protein stability due its net dissociation form the stabilizing SERCA2 complex in the SR.

Figure transgenic mice, which have more activity and protein in the heart, showed an antithetic alteration in PLB compared to the Specifically, PLB phosphorylation was reduced in the heart, while total protein was increased by 2. (panels A-D). The observed state of PLB, in conjunction with an increase in total protein, would significantly inhibit SERCA2 activity. Thus, overexpression of reduces cardiac contractility. The pentameric form of PLB is shown to demonstrate the shift in protein migration.

Figure 14. Alterations in PLB phosphorylation should directly alter SERCA2 function, thus effecting calcium loading within the sarcoplasmic reticulum and the magnitude of the calcium transient. Adult cardiac myocytes isolated from PKCa-l-mice showed enhanced calcium transients, suggesting greater calcium loading within the sarcoplasmic reticulum (panel A). Fura- 2 loaded PKCa-l-cells demonstrated a 52% increase in the peak release of calcium, as well as 17% faster calcium re-uptake corresponding to a 20% reduction in the time constant Tau cells from 6 wildtype mice and 33 cells from 4 PKCa-l-mice) (panel B). These data are consistent with enhanced SERCA2a function and larger calcium loads within the sarcoplasmic reticulum, thus reflecting a hyperdynamic state of the myocardium.

Figure 15. These results suggest that the augmented calcium transient observed in /-cardiac myocytes is due to increased loading of calcium within the sarcoplasmic reticulum.

Direct measurement of peak calcium release induced by caffeine administration in Indo-1-loaded cardiomyocytes demonstrated significantly greater sarcoplasmic reticulum calcium loads from PKCa-l-mice compared with wildtype (Wt) controls (P<0.05) (panels A shows a representative calcium tracing, B shows the quantitative data on peak caffeine-induced calcium release).

Figure 16. The observed increase in the calcium transient presented in Figure 14 could also be due, in part, to augmentation in L-type calcium current within the However, direct measurement of density did not vary between wildtype and PKCa-l-cardiac myocytes.

Figure 17. The alterations observed in PLB phosphorylation, without a corresponding change in receptor signaling or protein kinase A activity suggested a potential role for a phosphatase that acts on PLB. To investigate this potential effector pathway, PP2A-specific phosphatase assays were performed from wildtype hearts and (N=4 hearts each). Total protein phosphatase activity was decreased approximately 18% in hearts, while activity was decreased by greater than 30% and PP2A-specific activity was not significantly different. These results indicate that loss is associated with a decrease in activity within the heart.

Figure 18. Reciprocal to the data shown in hearts, overexpressing transgenic mice showed a significant increase in PP1 activity in the heart, but no change in PP2A activity. These results indicate that increased activity within the heart is associated with a specific increase in activity. Data are expressed as the relative phosphatase activity.

Figure 19. Consistent with the data presented in Figures 17 and 18, acute adenoviral infection of

cultured cardiomyocytes showed a 60% reduction in PP1 activity with expression of a dominant negative mutant, and a 30% augmentation in activity with wildtype overexpression (from triplicate experiments). That acute alterations in correspond with an alteration in activity suggests that might directly regulate activity, through a mechanism that will be elaborated in subsequent figures.

Figure 20. activity is regulated by a class of inhibitory proteins, such as inhibitory protein-1 I-1 directly binds PP1 resulting in the inhibition of activity, although ability to bind PP1 depends on its phosphorylation status from inducible signals. To examine the hypothesis that might directly phosphorylate thus regulating its association with an in vitro phosphorylation experiment was performed with bacterial generated and purified PKC in the presence protein was directly phosphorylated in vitro in a time-dependent manner at stoichiometric levels by PKC. Analysis of putative PKC phosphorylation sites within I-1 revealed a consensus motif at serine-67. Recombinant S67A mutant protein showed approximately 50% less phosphorylation by PKC compared with equal amounts of wildtype protein.

Figure 21. To further examine the potential mechanism whereby PP1 activity was altered by a series of I-1 immunoprecipitation experiments was performed from adenoviral- infected cardiomyocytes subjected to pull-down followed by western blotting (the input lanes were not immunoprecipitated). The data demonstrate that wildtype overexpression specifically reduced the ability of to interact with by approximately 50%, while dominant negative (dn) augmented complex formation by greater than 70%. Total levels did not vary in each of the immunoprecipitation reactions.

Figure 22. The ability of to interact with and inhibit is also regulated by protein kinase A-mediated phosphorylation of threonine-35 in Phosphorylation at this site renders a more potent inhibitor of PP 1, thus reducing its activity, opposite to the effect associated with phosphorylation of serine-67 by We investigated the effect of wildtype or dominant negative expression on I-1 phosphorylation at either threonine-35 or serine-67 using phospho-specific antibodies generated against each site. Cultured cardiomyocytes were infected with (Ad = adenovirus) to increase the sensitivity of the assay, together with or No change in threonine-35 phosphorylation was observed in response to modulation. However, AdPKCa-dn expression significantly decreased serine-67 phosphorylation by more than 70%, while augmented phosphorylation by greater than 60%.

Figure 23. The data presented in Figures 20-22 were extended in vivo using transgenic and gene-targeted mice in which endogenous I-1 phosphorylation was analyzed from heart extracts. Western blotting with serine-67 antisera demonstrated a significant reduction in phosphorylation from PKCa-/- hearts (more than 50%), while transgenic hearts had increased phosphorylation (2-fold) (P<0.05).

Figure 24. Consistent with the data shown in Figures 20-23, western blotting with protein extracts obtained from dilated failing human hearts also showed an increase in serine-67 phosphorylation compared with normal donor human hearts (panel B). These data are also consistent with a general increase in protein levels as assessed by western blotting from the same protein extracts (P<0.05) (panel A). These results indicate that failing, cardiomyopathic human hearts show an increase in levels and serine 67 phosphorylation in 1-1.

Figure 25. Confocal of protein in adult rat cardiomyocytes in culture shows PMA-induced translocation to the membrane and In unstimulated cells, is localized throughout the cell, but acute stimulation with PMA causes a rapid translocation to structures that are coincident with an enrichment of PLB and SERCA2 at the z- line within the sarcoplasmic reticulum. These data indicate that once activated, translocates to the proper intracellular localization to affect calcium handling within the sarcoplasmic reticulum.

Figure 26. Here we tested the hypothesis that the relatively mild hypercontractile status observed in gene-targeted mice might benefit a failing heart. PKCa-/-mice and wildtype littermate controls were subjected to long-term aortic banding-induced heart failure. Mice underwent TAC within the thoracic cavity beginning at 8 weeks of age for a period of 12 weeks, after which cardiac function was assessed by working heart preparation. Hearts from wildtype mice showed a reduction in maximal dP/dt and a 35% reduction in LVP compared to sham operated controls of the same age, while null mice did not show a significant decrease in either parameter (N=4 hearts in each cohort). These results indicate that are resistant to pressure overload-induced cardiac decompensation and loss of contractility.

Figure 27. Echocardiography was also performed to further examine contractility affects following long-term aortic banding-induced heart failure as described in Figure 26. null mice and wildtype controls were subjected to TAC within the thoracic cavity beginning at 8 weeks of age for a period of 12 weeks, after which cardiac function was assessed by echocardiography. Hearts from wildtype mice subjected to 12 weeks of TAC showed a more prominent increase in left ventricular end diastolic (LVED) and systolic (LVES) ventricular chamber dimensions compared with to the same stimulus (panel A). Cardiac left ventricular fractional shortening was also more prominently depressed in wildtype TAC mice compare with which showed much less loss of ventricular performance (panel B). These results further indicate that are resistant to pressure overload- induced cardiac and loss of contractility in vivo.

Figure 28. A mouse model of dilated cardiomyopathy due to ablation of the muscle lim protein (MLP) gene was also analyzed as a second heart failure model. By echocardiography, 2 month-old MLP null mice showed reduced functional capacity and greater left ventricular chamber dilation compared with wildtype controls or (panels A, B). However, MLP null mice that were null for showed a significant improvement in heart failure symptoms, such as less ventricular dilation (LVED and LVES) and preserved fractional shortening (panels A, B). These results indicate that loss of prevents cardiac dysfunction and remodeling in another mouse model of cardiomyopathy and heart failure in vivo.

Figure 29. was also assessed in using the isolated ex vivo working heart preparation. These data showed a significant reduction in cardiac contractility in that was prevented in mice that were also null for (double nulls), as measured by changes in maximum or left ventricular pressure developed (LVP). These results further indicate that loss of prevents cardiac dysfunction in the MLP mouse model of cardiomyopathy and heart failure ex vivo.

Figure 30. The prevention of heart failure and reductions in cardiac contractility by deletion of within the MLP-/-background suggested that other aspects of cardiomyopathy might be reduced. Compared with the double null mice (also missing showed a loss in reactive hypertrophy that typifies the MLP null phenotype. Thus, enhancing contractility through deletion prevented the manifestations of dilated cardiomyopathy related to increases in heart weight (HW) to body weight (BW) ratio increases.

Figure Consistent with the data presented in Figure 30, single MLP-/-mice had histological disease associated with a dilated and enlarged myocardium in longitudinal section, while the double null mice (also missing showed essentially no pathology. These results further support the contention that deletion prevents the manifestations of dilated cardiomyopathy related to histopathology and gross morphological changes.

Figure 32. Transgenic mice expressing 3-fold more PP1 catalytic subunit in the heart are known to have reduced functional capacity and cardiomyopathy by 3 months of age. Given our data that suggest can directly regulates activity in the heart (Figures 18-24), we reasoned that loss of would partially inhibit the increased activity associated with moderate overexpression of null mice crossed with PP1 transgenic mice demonstrated a significant reduction in PP1 activity in the heart. Hearts derived from PP1 transgenic mice showed an approximate increase in activity of 2.5 fold compared with hearts from

wildtype mice. Once again, hearts from showed a significant reduction in cardiac activity. No change in PP2A was observed. These results indicate that loss reduces the effectiveness of overexpressed PP1 in the heart.

Figure 33. By 3 months of age, transgenic mice showed significant reductions in ventricular performance as assessed by echocardiography. However, deletion within the transgenic background, which was shown in Figure 32 to reduce activity effectively prevented the loss of ventricular performance. These results indicate that can prevent cardiomyopathic effects and contractile deficits observed in PP1 transgenic mice in vivo.

Figure 34. At 3 months of age, transgenic mice also have reduced contractility as measured with an ex vivo working heart preparation. However, deletion within the transgenic background, similarly prevented the loss of contractility as measured by changes in maximum minimum and left ventricular pressure developed (LVP). These results further support the conclusion that can reverse the cardiomyopathic effects and contractile deficits observed in transgenic mice ex vivo.

Figure 35. To demonstrate the benefits inhibition on mortality resulting from heart failure and cardiomyopathy, death was also quantified as an end-point. The 12 week TAC experiment described in Figures 26 and 27 was also monitored for animal deaths in both control wildtype mice, as well as PKCa-/-mice. The data show that significantly more deaths were observed in wildtype mice subjected to TAC over the 12 week time course of TAC compared with subjected to TAC. No deaths were observed in sham control mice of either genotype. These results indicate that loss protects mice from TAC-induced heart failure and ultimately, an untimely demise. In addition, mortality was also assessed in the MLP-/-mice.

Figure 35B indicates that MLP-/-mice have a high mortality rate compared to the other groups presented. The high mortality rate in is attenuated in mice lacking both MLP and (double nulls). The data indicate that in the setting of MLP ablation and heart failure, provides a survival benefit.

Figure 36. To more carefully assess the potential role as a regulator of cardiac contractility we used acute administration to wildtype or PKCa-/-hearts in the ex vivo working heart preparation. The PKC-activating class of compounds referred to as phorbol esters were employed to elicit acute, PKC-dependent alterations in cardiac contractility. Here isolated hearts were infused with PMA at 9 different concentrations ranging from M (panels A, B). The data show that acute PMA infusion has essentially no effect on the contractile performance of wildtype mouse hearts at concentrations ranging from $8x10-1$'through M, with respect to either Maximum dP/dt or Minimum (panels A, B). However, concentrations higher than M produced a marked decrease in functional performance in wildtype mouse hearts, suggesting that PKC activation could reduce cardiac contractility in this preparation (panels A, B). However, null hearts subjected to the same concentrations of PMA showed an immediate positive inotropic effect to low doses of PMA, and only a mild depression in functional performance at the highest concentrations of PMA (Figure 36 A, B).

These results indicate that PMA-induced depression of cardiac contractility directly depends on Such data support a critical role for as an acute negative regulatory of contractility, distinct from other PKC isozymes that are also activated by PMA.

Figure 37. Since may serve as a novel target for altering cardiac contractility acutely, and hence affect heart failure, it was of interest to examine the relative abundance of versus the other classic PKC isozymes from the human heart. Recombinant human protein standards (generated in bacteria) were purchased from a commercial vendor so that a standard curve of protein content versus signal intensity

by western blotting could be generated for pI, and These protein standards were run on the same gel and subjected to antibody detection by western blotting as whole cell protein samples derived from 6 normal human hearts (panels A, B). The data demonstrate that expressed at significantly higher levels than the other PKC isozymes that were analyzed in the human heart (Panels A, B). These results suggest that PKC. is a prominent PKC isoform in the human heart Figure 38. The relatively selective inhibitory compound Ro-32-0432 yl) maleimide, Salt] was used as a means of directly examining the effects of acute inhibition on cardiac function and contractility using an ex vivo working heart preparation. Adult wildtype mouse hearts were infused vehicle control (10% DMSO) or Ro-32-0432 in 10% DMSO at concentrations ranging between through values throughout the concentration time course (7 minutes per 10 different incremental concentrations) were summated for statistical purposes, representing an average dosage M. The vehicle control and experimental groups showed heart rates of 363 +/-15 and 295 beats per minute, respectively, before any treatments were begun. The average heart rate of the vehicle and drug treated groups was and 292 +/-6 beats per minute, respectively. Despite the lower heart rate, the Ro-32-0432 infused group showed a 20% increase in acute contractile function, measured as maximum dP/dt, and a 20% increase in left ventricular pressure developed 05) (panels A, B). Four animals were analyzed in the Ro-32-0432 group and compared with three animals in the vehicle control group. The approximate 20% change in acute contractile performance in the Ro-32-0432 treated group is similar to the increase in cardiac function observed in null mice discussed earlier in this application. Collectively, these results indicate the acute inhibition of using Ro-32-0432, effectively augments cardiac function and contractility.

Figure 39. PKC isozyme translocation is often associated with activation, and PKC inhibitory agents can block this translocation event. A PKCa-green fluorescent protein fusion expressing adenovirus was generated as a means of carefully monitoring translocation, or inhibition of translocation in neonatal cardiomyocyte cultures. In response to vehicle treatment (DMSO), was unaffected compared to untreated, showing a fairly diffuse localization throughout the cell, with a mild sarcomeric organization. However, 60 minutes of PMA stimulation caused a robust redistribution of so that the diffuse background of localization was replaced with a highly localized and punctate staining pattern with less overall fluorescence. The net effect of such redistribution is a change in local fluorescence characteristics in each cell, which could be easily detected in a large-scale screening assay. Thus, the appropriate inhibitory compound could be quickly identified based on cellular redistribution.

Figures 40A and 40B. In order to demonstrate the ability of a inhibitor to 10, 14,15-tetrahydro-4, 9: 16, 21-dimetheno-1H, 13H-dibenzo [E, K] pyrrolo- [3, 4- H] [1, 4,13] 3 (2H)-dione 32,1045- 1052), was administered in normal rats as described in the experimental section. was dissolved in 20 % Sulfobutyl ether-B-cyclodextrin sodium salt (Captisol) in a 50 mM acetate buffer at pH 5. 0. The compound was infused for 5 minutes at each concentration in Figure 40.

At the 1000 nmol/kg/min dose, demonstrated a significant increase in maximum dP/dt (Figure 40A) and minimum dP/dt (Figure 40B). Figures 40A shows maximum dP/dt and Figure 40B shows minimum dP/dt including no compound (baseline; and following infusion of 0.1, 0.3, 1,3, 10,30, 100,300 and 1000 nmol/kg/min of which are indicated on the abscissa. The drug was then stopped for 5 minutes (P/D) and dobutamine (Dob) was administered at 5.0 for 5 minutes. In Figure 40B, values for minimum dP/dt are expressed as the absolute or numeric value for simplicity. At the dose, maximum dP/dt was increased while minimum increased 17% compared to baseline measurements, consistent with the data in the null mice and administration of Ro-32-0432 in isolated work-performing heart preparations. Figure 40A and 40B, the asterisk indicates a statistically significant difference (P<0.05) from baseline values, as determined by a one-way analysis of variance (ANOVA) with a Dunnett's Multiple Comparisons post-hoc test. This increase in cardiac contraction and relaxation at 1000 occurred in the absence of effects on heart rate (Baseline: beats per minute vs. 1000 beats per minute, not statistically significant) or left ventricular systolic blood pressure (Baseline: mmHg vs. 1000 : beats per minute, not statistically

significant). These data indicate that inhibition in normal rats result in positive cardiac inotropy (contraction) and lusitropy (relaxation).. LY338522, an active metabolite of LY333531, has also shown to be effective in inhibiting the PKC isoforms (Burkey JL et al. (2002) Xenobiotica. 32,1045-1052).

Figure 41. In order to demonstrate the efficacy of in vivo in a myocardial infarction model, Ro-31-8220, indol-3-yl) maleimide, Bisindolylmaleimide IX, Methanesulfonate, a known PKC inhibitor (Han Z et al. (2000) 7,521-530) was infused in rats which underwent surgery to induce a myocardial infarction (MI rats). was dissolved in 20 % Sulfobutyl ether-B- cyclodextrin sodium salt (Captisol) in a 50 mM acetate buffer at pH 5.0. Ro-31-8220 was delivered in vivo as described in the experimental section. Infusion of Ro-31-8220 resulted in a dose dependent enhancement of the percent increase in maximum which reached statistical significance (P<0.05) at the 300 dose (Figure 41; One-way ANOVA and Dunnett's Multiple Comparisons test). Figure 41 shows the percent increase in maximum dP/dt from baseline following infusion of 10,30, 100,300 and 1000 nmol/kg/min of Ro-81-8220, which are indicated on the abscissa. The drug was then stopped for 5 minutes (P/D) and dobutamine (Dob) was administered at 5.0 for 5 minutes. These data indicate that inhibition of results in a positive inotropic effect in a rat model of heart failure. The inotropic benefit observed with infusion of was greater than that seen with dobutamine, a clinically administered inotrope in ADHF. These data suggest that delivery of a inhibitor to human's inflicted with myocardial dysfunction in contraction or relaxation, such as that observed in ADHF, would provide a functional benefit in these patients, a desired outcome of medical treatment.

Except as otherwise noted, all amounts including quantities, percentages, portions, and proportions, are understood to be modified by the word"about", and amounts are not intended to indicate significant digits.

Except as otherwise noted, the or more".

All documents cited are, in relevant part, incorporated herein by reference; the citation of any document is not to be construed as an admission that it is prior art with respect to the present invention While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope.

US2004030581_31032005_pf_000058.g4" LX="0" LY="0" TI="PA"/>

WE CLAIM: 1. A transgenic mouse comprising at least one stably incorporated expression cassette in the genome of at least one cell, said expression cassette comprising a cardiac tissue-preferred regulatory sequence operably linked to a nucleotide sequence of interest selected from the group consisting of : (a) the nucleotide sequence of SEQ ID NO: 1; (b) a nucleotide sequence having at least about 90% identity to the nucleotide sequence of SEQ ID NO: 1; (c) a nucleotide sequence encoding a polypeptide having the amino acid sequence of SEQ ID NO: 2; (d) a nucleotide sequence encoding a polypeptide having at least about 90% identity to the amino acid sequence set forth in SEQ ID NO: 2; (e) a nucleotide sequence comprising at least about 50 contiguous nucleotides of a nucleotide sequence set forth in SEQ ID NO: US2004030581_31032005_pf_000058_0001.g4" LX="1017" LY="1259"> ; US2004030581_31032005_pf_000058_0002.g4" LX="559" LY="1328"> a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence set forth in SEQ ID NO: 1; (g) the nucleotide sequence set forth in SEQ ID NO: 7; and (h) a nucleotide sequence consisting of a complement of any one of the nucleotide sequences in (a), (b), US2004030581_31032005_pf_000058_0003.g4" LX="792" LY="1590"> (d), (e), or (g).

2. The transgenic mouse of claim 1, wherein the expression cassette comprising a cardiac tissue-preferred regulatory sequence is operably linked to the nucleotide sequence of SEQ ID NO: 1.

3. A method for identifying compounds that modulate cardiac contractility, comprising the steps of : (a) contacting a compound with protein kinase C-a protein; (b) determining whether the compound binds protein kinase US2004030581_31032005_pf_000058_0004.g4" LX="1456" LY="2208"> ; and (c) identifying those compounds that bind protein kinase C-a as modulators of cardiac contractility.

4. A method for identifying compounds that modulate cardiomyopathy, comprising the steps of : (a) contacting a compound with protein kinase US2004030581_31032005_pf_000058_0005.g4" LX="1250" LY="2616"> protein; US2004030581_31032005_pf_000059.g4" LX="0" LY="0" TI="PA"/> (b) determining whether the compound binds protein kinase US2004030581_31032005_pf_000059_0001.g4" LX="1482" LY="388"> ; and US2004030581_31032005_pf_000059_0002.g4" LX="543" LY="459"> identifying those compounds that bind protein kinase C-a as compounds as modulators of cardiomyopathy.

5. The method of claim 3 or claim 4, wherein the protein kinase US2004030581_31032005_pf_000059_0003.g4" LX="1420" LY="647"> protein is expressed in a cell and the effect of the modulator is measured as change in the protein kinase C-a activity as compared to a cell that is not contacted with the compound.

6. The method of claim 3 or 5, further comprising the steps US2004030581_31032005_pf_000059_0004.g4" LX="1345" LY="936"> (a) selecting those compounds that modulate activity of protein kinase US2004030581_31032005_pf_000059_0005.g4" LX="1644" LY="0"> protein, and further determining whether those compounds modulate cardiac contractility in a cardiac contractility model system; and (b) identifying those test compounds that modulate cardiac contractility in the cardiac contractility model system as candidate compounds for modulating cardiac contractility.

7. The method of claim 4 or 5, further comprising the steps US2004030581_31032005_pf_000059_0006.g4" LX="1345" LY="1411"> (a) selecting those compounds that modulate activity of protein kinase US2004030581_31032005_pf_000059_0007.g4" LX="1644" LY="0"> protein, and further determining whether those compounds modulate cardiomyopathy in a cardiomyopathy model system; and (b) identifying those test compounds that modulate cardiomyopathy in the cardiomyopathy model system as candidate compounds for modulating cardiomyopathy.

8. The use of the transgenic mouse of claim 1 or 2, or a cell or tissue thereof, as the model system in the method of any of claims 5,6, or 7.

9. A transgenic mouse comprising at least one disrupted protein kinase US2004030581_31032005_pf_000059_0008.g4" LX="1527" LY="2086"> gene in the genome of at least one cell, the disruption being sufficient to decrease protein kinase C-a expression levels.

10. The use of a protein kinase C-a modulating compound in the manufacture of a medicament for treating or preventing a disorder modulated by cardiac contractility in a mammal.

11. The use of a protein kinase US2004030581_31032005_pf_000059_0009.g4" LX="900" LY="2563"> modulating compound in the manufacture of a medicament for treating or preventing a cardiomyopathy in a mammal. US2004030581_31032005_pf_000060.g4" LX="0" LY="0" TI="PA"/>

12. The use of a protein kinase C-a modulating compound in the manufacture of a medicament for treating or preventing an acute heart failure in a mammal in need thereof.

13. The use of a protein kinase C-a modulating compound according to any of claims 10, 11, or 12, wherein the protein kinase US2004030581_31032005_pf_000060_0001.g4" LX="880" LY="743"> modulating compound is a protein kinase C-a inhibitor selected from the group consisting US2004030581_31032005_pf_000060_0002.g4" LX="849" LY="800"> LY333531 and Ro-31-8220.

14. A method of treating or preventing a disorder modulated by cardiac contractility in a mammal in need thereof, comprising: (a) identifying an animal in need of treating or preventing a disorder modulated by cardiac contractility; and (b) administering a protein kinase C-a modulating compound to said mammal.

15. A method of treating or preventing cardiomyopathy in a mammal in need thereof, comprising: (a) identifying an animal in need of treating or preventing cardiomyopathy; and (b) administering a protein kinase C-a modulating compound to said mammal.

16. A method of treating or preventing acute heart failure in a mammal in need thereof, comprising: (a) identifying an animal in need of treating or preventing acute heart failure; and (b) administering a protein kinase US2004030581_31032005_pf_000060_0003.g4" LX="966" LY="1889"> modulating compound to said mammal.